SOIL SCIENCE

RUTGERS COLLEGE.

VOL. I. NEW BRUNSWICK, N. J., FEBRUARY, 1916. No. 2.

THE ACTINOMYCES OF THE SOIL.*

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The study of soil microörganisms has attracted the attention of many investigators, and great advance has been made in the study of bacteria, fungi, and protozoa. Very little attention, however, has been paid to the actinomyces as a group of soil organisms. The present work has been undertaken with the purpose of demonstrating the occurrence of actinomyces in different soil types, at different depths, and under different cultural and climatic conditions. An attempt has been made to secure a knowledge of the physiological activities of these organisms and their possible part in soil fertility.

During the past forty years about forty types of actinomyces have been studied under different names and under varied environmental conditions. Most of these descriptions are so incomplete that it was found impossible to identify many of the organisms at hand with those previously studied. Almost all former investigations have been undertaken from a pathological standpoint, and the descriptions have been adapted to that purpose. With one exception, the studies heretofore made on soil actinomyces have been limited to a very few representatives of this group.

The present paper records an attempt to study the occurrence of actinomyces in the soil, and to classify them according to their morphological and physiological characters; it is necessarily incomplete, inasmuch as the sources of literature for study and identification are so limited and the field of investigation is so large.

HISTORICAL.

Cohn (5), in 1875, was the first to study an actinomyces, under the name of "Streptothrix" Foesteri. Bollinger (2) found one in an "actinomycose" swelling of cattle, and named it Actinomyces for its radiating form. Rossi-Doria (19) studied *Streptothrix alba, Str. nigra (Str. Foes-*

* Received for publication January 15, 1916.

teri), *Str. albido-flava, Str. violacea, Str. carnea* and *Str. aurentiaca.* Gasperini (9) made an important contribution to the knowledge of actinomyces by his work on *Act. albus, Act. sulphureus, Act. luteo-roseus, Act. asteroides, Act. carneus* and *Act. aurentiaca.* More work followed: names were mixed and the terms "Actinomyces," "Streptothrix," "Cladothrix" and "Oöspora" were often interchanged, all of them meaning the same type. Petruschky (18) united all fine mycelial, unseptate fungi into the family *"Trichomycetes,"* and divided this into four groups : (1) Actinomyces, those forming a radiating growth when parasitic in animal tissues, (2) Streptothrix, having an abundant true branching, wavy or curly growth, late fragmentation and formation of conidial chains, which serve as organs for multiplication, (3) Cladothrix, having false branching, quick fragmentation and therefore bacillary character of old cultures, (4) Leptothrix, never shows true branching, but stiff, little curved hyphae on which no organs of division can ever be detected.

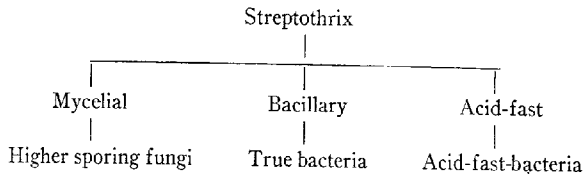
Lehmann and Newmann (14) consider the actinomyces a special group, which stands between the Hyphomycetes and Schizomycetes; related to the latter by their slender hyphae and protoplasmic properties, and to the former by the branching formation of aerial hyphae with conidia-like structures. They are defined as "delicately threaded organisms, with true branching, in part very abundantly ramifying mycelium, partly with the formation of conidia. There is a tendency to the formation of clubs or knobs at the ends of threads." This family is divided into two groups. Group I contains the corynebacteria (L & N), which are "slender, often somewhat bent rods, usually having a tendency toward a clubbed swelling at the ends, branches rarely observed in young cultures, easily broken off; always non-motile, conidia never found"; and Mycobacteria (L & N), with "clubbed swellings rare in cultures, in tissues somewhat more common, staining with difficulty or not at all." Group II, the Actinomyces, are described as follows by Hartz: "Mycelial threads long, thin; extending or winding; dividing without partitions, with delicate sheaths and true branching; many species separate from the hyphae rows of short spores (conidia) which, whitish and mold-like, project upward above the solid nutrient substratum. Motility sometimes manifested. Almost all varieties emit a musty odor."

Sanfelice (21) very ably pointed out the faults in Petruschky's classification. He showed that actinomyces are, according to morphology and properties as revealed in culture, true and proper Streptothrices. The peculiarity, which seemed of so much importance to Petruschky that he separated the Actinomyces from the Streptothrix types, is only a specific property. The term "Actinomyces" as understood by Sanfelice, and by Lehmann and Newmann, will be used by the authors, since this term alone

can be applied to the great mass of the soil microörganisms which are discussed in this paper.

Musgrave (17) and others used the name Streptothrix for the genus. They define Streptothrix "as branching filamentous organisms, which develop into colonies made up of organisms and their transformation products. Terminal hyphae may or may not be radial, may or may not have clubs. This group is in general gram positive and many are acid fast." Foulerton (7) describes Streptothrix as "a tangled mass of branching mycelium. The mycelial stage is followed by segmentation and fragmentation, producing bacillary forms; and in artificial media by chain sporulation."

Claypole (4) gives the following tentative outline for the development of the microörganisms from the Streptothrix group:



She says, "The limits of species variation of the Streptothrices are neither set nor well known. The cause of confusion and for diverse opinions and practices lies in the extreme morphological and biological variability of these fungi. Some strains grow feebly on all media; some only on special media, and some apparently cannot be cultivated. Much variation is to be found in the morphology of any given organism. It changes its appearance with differing culture media. The well known granules or 'Drusen,' a branched mycelial mass, fragmentations into apparent bacilli and cocci, true spores as well as the minute structures left after chain sporulation, may be found in the life history of one species. It would seem biologically more reasonable to look upon this group of Streptothrices with their variable morphology and close relationships, as representing the ancestral type that gave both the higher fungi and the true bacteria and not as being themselves, higher bacteria."

Rullmann (20), the first to study the actinomyces in the soil, concludes that *Act. odorifer* causes the soil odor, which it also forms on media containing carbohydrates, but that it has no nitrifying ability.

Beijerinck (1) studied the existence of actinomyces in nature and their ability to form "quinone," an oxidizing agent. He found actinomyces in garden soil even at a depth of one meter, and in dune sand as deep as two meters; he found them also on the roots of many plants. He states that they inhabit the outside cells of the plants as saprophytes,

not as parasites. They are omnivorous. They can live on media free from combined nitrogen, which they get from the air or distilled water (not atmospheric nitrogen), so small is their nitrogen requirement. They must play an important, if not a dominant, part in humus formation. He brought out the peculiar property of the actinomyces, namely, their power to reduce nitrates to nitrites without causing much loss of nitrogen. He also found that subsoils, though containing fewer numbers of actinomyces than the surface soil, are relatively richer in these than in other microörganisms. The only types studied by Beijerinck were *Act. albus* and *Act. chromogenus.*

Hiltner and Störmer (12) found that in the spring actinomyces form 20 per cent of the total bacterial numbers in the soil, while in the fall they form 30 per cent.

Fousek (8) found in the fall a greater percentage of actinomyces than in the spring. He found them to form 20 to 30 per cent of the organisms in loam soils, 8 to 15 per cent in clay soils, and 7 to 10 per cent in sands. Fallow soils contained larger numbers than cultivated soils. The actinomyces assimilate nitrates, ammonia and amido nitrogen, and form ammonia from organic substances. Nitrates are reduced to nitrites; free nitrogen is not assimilated. He finds *Act. albus* and *Act. chromogenus* to be predominant among the soil actinomyces.

Hagem (10) isolated four actinomyces from the soil, but from his short description of the macroscopic appearance of the organisms, one can hardly get a true idea as to which species he really had.

Münter (16) isolated seven organisms from different soils: *Act. odorifer, Act. chromogenus, Act. albus* I and II, and three more organisms which he terms *Act. S-a, Act. S-b,* and *Act. S-c.* He finds that these organisms can assimilate sugar and organic salts; and that organic substances have a strong influence on pigment production. The organisms are very sensitive to acids and alkalies. All of them liquefied gelatin, with or without the production of a brown pigment.

Conn (6) found that actinomyces may make up as much as 40 per cent of the soil bacteria.

The most complete work on the actinomyces of the soil is that of Krainsky (13), who has given a full description of eighteen well characterized and defined species of soil actinomyces. The organisms have been studied on different media and rather complete morphological and physiological qualities are recorded. All of them reduce nitrates to nitrites to a greater or less extent. Krainsky studied the production of enzymes, as did Münter and several others. Some of Krainsky's organisms are reported as strong cellulose destroyers. On allowing plates of calcium malate agar to incubate for thirty days he found 20,800 colonies of actino-

myces per gram of soil, which was 30 per cent of the total number of organisms developing on this medium. The upper soil layer was found to be poorer in fast growing forms than the soil at the depth of fifty centimeters. Krainsky concludes that the actinomyces play an important part in the decomposition and humification of plant remains in the soil.

EXPERIMENTAL.

I. Methods of Study.

1. *Soils used.*

Seven soils have been considered in this work. They represent different types, from several localities and under differing climatic and cultural conditions. The principal purpose was to determine whether there are any so-called soil actinomyces, whether organisms isolated from one locality are found in another, and whether there is a constancy in the occurrence of the particular species. Three soils from the eastern Los Angeles County, California, were among those used. The samples from these represent a composite of the surface eight inches. They are: (1) an upland, residual, loam unirrigated and cropped for grain and hay, which will be designated as "upland soil"; (2) a heavy adobe soil, irrigated, from an orange orchard, termed "adobe soil"; (3) a sandy loam, irrigated, also from an orange orchard, termed "California loam." A fourth soil was secured from the experimental farm of the Oregon Agricultural College at Corvallis, Oregon. This is an adobe type and has been cropped to legumes and small grains, termed "Oregon adobe." The three remaining types were taken from the experimental grounds of the New Jersey Agricultural Experiment Station at New Brunswick, N. J., (5) a Sassafras sandy loam, heavily manured every year, under garden crops, and termed "garden soil"; (6) a Sassafras sandy loam unmanured for the past twenty years, under orchard, termed "orchard soil"; and (7) a heavy clay soil, under permanent meadow, termed "meadow soil." Samples from the last three soils have been taken at depths of 1, 4, 8, 12, 20 and 30 inches. All sampling has been done under sterile conditions, and in the subsequent handling and plating the usual bacteriological precautions against air or other contaminations have been observed. The New Jersey soils were plated out within a few minutes after the samples were taken. In the case of the Oregon and California soils the necessary time for shipment of course elapsed between sampling and plating.

2. *Media used.*

Most investigators have used beef extract agar and gelatin for the study of actinomyces. As was pointed out before, the actinomyces will grow readily on any medium containing enough carbohydrates. Their nitrogen requirements are very small. Beef extract agar and gelatin are

not suitable media for the culture of actinomyces, because, first, they are rich in nitrogen and for this reason do not bring out the characteristic colors of the organisms, aerial mycelium is not readily formed, and when it is produced, is of a chalky color; furthermore, most species tend to produce colonies more or less white in color accompanied by a brown pigment in the substratum. Second: These media are not constant in composition, and the growth and color of the actinomyces, two of the most important factors in their differentiation, are very sensitive to change in the composition of the medium. The above probably accounts for the fact that early investigators reported one *Act. albus* and one *Act. chromogenus* in the soil.

Krainsky has used in his work a calcium malate agar and several other synthetic media. For this, as in any bacteriological work, media of constant chemical composition are desirable. As actinomyces may change considerably in color production and character of growth from the mere process of transferring several times on the same medium, it can be readily seen that different media, as well as those of varying composition, would give very incomparable results.

Brown's (3) albumen agar, slightly modified, has been used for the isolation of the organisms from the soil. Suitable dilutions, varying from 1:200,000 for the surface soils to as low as 1:10,000 for the deeper subsoils, were used. The plates were allowed to incubate for 14 days at 22° C. Counts were then made and the actinomyces transferred to Czapeck's solution agar. On this latter medium macroscopic and microscopic studies of all the organisms were made. Each organism was also studied on potato plugs (incubated at 30° C.) and on 15 per cent gelatin, in distilled water (incubated at 15° to 16° C.). Some species were studied on Czapeck's solution, where a characteristic growth is produced; and on 1 per cent dextrose broth, for gas production. A few were studied on beef extract agar, mannite agar, and several other media.

All the actinomyces studied liquefy gelatin. They seem able to get all necessary food from the pure gelatin in distilled water, decomposing the gelatin in all probability by means of an enzyme. The various species show marked differences in the rapidity with which they liquefy gelatin. Some form a liquid ring of 1 to 2 cm. diameter in three days; others hardly form a liquefied circle of 2 mm. diameter in ten days. There is also a difference in color production on gelatin. In this connection the actinomyces could be divided into two groups: those that do not produce any color on gelatin, the liquefied portion remaining pure white; and those that produce a pigment (usually brown) in and around the liquefied portion. The depth of the pigment varies somewhat in the different species. Some of the organisms produce aerial mycelium on the gelatin. This appears to be characteristic of the particular species.

3. *Numbers of Actinomyces in the Soil, and Their Relation to Numbers of Bacteria.*

One of the authors (23) has pointed out the fact that, though the numbers of actinomyces decrease with soil depth, their numbers, relative to those of bacteria and fungi, greatly increase. At the depth of one inch the actinomyces made up from 7.3 to 12.1 per cent of the total number of microörganisms; at the depth of 30 inches they constituted 52.7 to 83.6 per cent of the total numbers.

Additional data are presented in the following tables.

TABLE I.

BACTERIA AND ACTINOMYCES IN NEW JERSEY SOILS.

Soil Depth in inches	Garden Soil				Orchard Soil				Meadow Soil			
	Bacteria		Actinomyces		Bacteria		Actinomyces		Bacteria		Actinomyces	
	Numbers	%	Numbers	%	Numbers	%	Numbers	%	Numbers	%	Numbers	%
	SEPTEMBER 15, 1915.											
1	7,870,000	93.7	533,000	6.3	7,000,000	92.9	533,000	7.1	8,600,000	90.9	867,000	9.1
4	6,400,000	87.3	933,000	12.7	6,200,000	88.6	800,000	11.4	7,200,000	85.0	1,267,000	15.0
8	3,670,000	93.2	267,000	6.8	2,930,000	92.6	233,000	7.4	3,933,000	86.8	600,000	13.2
12	1,867,000	93.0	140,000	7.0	1,353,000	90.6	140,000	9.4	767,000	81.6	173,000	18.4
20	320,000	62.4	193,000	37.6	140,000	52.6	126,000	47.4	320,000	63.2	187,000	36.8
30	113,000	31.4	247,000	68.6	53,000	29.6	126,000	70.4	153,000	50.0	153,000	50.0
	NOVEMBER 2, 1915.											
1	4,700,000	87.0	700,000	13.0	4,600,000	88.5	600,000	11.5	15,400,000	92.2	1,300,000	7.8
4	4,500,000	85.0	800,000	15.0	4,500,000	77.6	1,300,000	22.4	7,000,000	86.4	1,100,000	13.6
8	3,500,000	74.5	1,200,000	25.5	1,560,000	76.5	480,000	23.5	1,710,000	79.9	430,000	20.1
12	720,000	62.1	440,000	37.9	670,000	59.0	470,000	41.0	1,040,000	79.4	270,000	20.6
20	210,000	42.0	290,000	58.0	130,000	19.4	540,000	80.6	690,000	67.0	340,000	33.0
30	160,000	24.0	510,000	76.0	90,000	16.4	460,000	83.6	160,000	44.6	200,000	55.6
	NOVEMBER 30, 1915.											
1	5,300,000	85.5	900,000	14.5	4,800,000	86.6	700,000	13.4	8,100,000	93.7	550,000	6.3
4	4,300,000	84.3	800,000	15.7	3,200,000	82.0	700,000	18.0	4,500,000	86.5	700,000	13.5
8	3,600,000	75.0	1,200,000	25.0	1,800,000	81.8	400,000	18.2	1,700,000	70.8	700,000	29.2
12	725,000	82.0	160,000	18.0	680,000	74.7	230,000	25.3	750,000	85.2	130,000	14.8
20	160,000	39.0	250,000	61.0	330,000	58.9	230,000	41.1	30,000	33.3	60,000	66.7
30	90,000	35.6	170,000	64.4	250,000	51.0	240,000	49.0	50,000	50.0	5,000	50.0

GENERAL AVERAGE FOR ALL THREE SOILS.

Soil Depth	Bacteria		Actinomyces	
	Numbers	%	Numbers	%
1 inch	7,340,000	90.8	743,000	9.2
4 inches	5,300,000	85.0	933,000	15.0
8 inches	2,710,000	81.6	612,000	18.4
12 inches	950,000	79.9	239,000	20.1
20 inches	259,000	51.3	246,000	48.7
30 inches	124,000	34.6	240,000	65.6

The data presented in Table I bear out well the observations of previous investigators, in regard to the distribution of actinomyces in the soil and their numbers, relative to those of bacteria.

The numbers of both actinomyces and bacteria are greatest in the surface soil, but while the bacteria decrease rapidly below a depth of four inches, the numbers of actinomyces are practically constant at depths from 8 to 30 inches. This point is graphically brought out in Fig. 1, where the average percentages of bacteria and actinomyces from Table I are plotted.

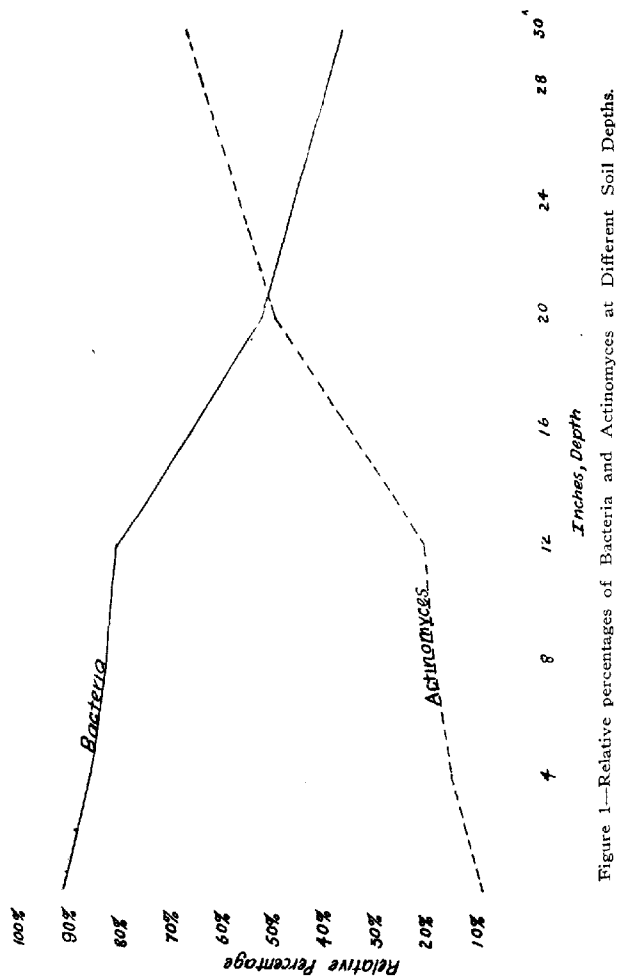


Figure 1—Relative percentages of Bacteria and Actinomyces at Different Soil Depths.

TABLE II.

BACTERIA AND ACTINOMYCES IN OREGON SOIL.

Depth	Bacteria		Actinomyces	
	Numbers	Per cent	Numbers	Per cent
0— 2 inches	13,100,000	84.6	2,400,000	15.4
3— 5 inches	10,600,000	63.1	6,200,000	36.9
10—14 inches	2,960,000	63.8	1,680,000.	36.2
18—22 inches	680,000	48.6	720,000	51.4
28—32 inches	800,000	64.6	440,000	35.4

Table II presents the numbers and percentages of actinomyces and bacteria in the "Oregon adobe" soil. A close resemblance is seen between the percentages of bacteria and of actinomyces when the figures are compared with those in Table I. This is particularly true of the "meadow soil" and the "Oregon adobe." As the climatic conditions in the two places are very similar and the two soils have much in common, e. g., fine texture, large water capacity and a high water table, the similarity in results was to be expected.

TABLE III.

BACTERIA AND ACTINOMYCES IN CALIFORNIA SOILS.

Soil	Bacteria		Actinomyces	
	Numbers	Per cent	Numbers	Per cent
Upland (Sample I)	1,935,000	80.4	380,000	19.6
Upland (Sample V)	2,310,000	55.0	1,890,000	45.0
Upland (Sample VI)	2,420,000	62.7	1,445,000	37.3
Sandy Loam	6,010,000	80.8	1,430,000	19.2
Adobe	3,620,000	78.0	800,000	22.0

Table III gives similar data obtained from the southern California soils. As was noted before, these samples were a composite of the surface soil to a depth of 8 inches. A comparison of these figures obtained from semi-arid soils with those from humid soils brings out striking differences. In the surface eight inches of the New Jersey soils studied, actinomyces make up an average of 14.2 per cent of the micro-flora, exclusive of the higher fungi; while in the California soils this average is 28.6 per cent. This seems to indicate that semi-arid surface soils are relatively much richer in antinomyces than are those of humid regions.

MORPHOLOGY OF THE ACTINOMYCES.

The actinomyces grow on artificial media in the form of small, slowly developing, usually round colonies, which are partly submerged and partly aerial. Most of them usually begin to develop from the bottom of the plate, slowly pushing upward their rounded, glossy surface, until, on reaching the surface of the medium, they usually become covered with an aerial mycelium, which reminds one of lower spore-forming fungi. The aerial mycelium forms aerial spores which serve for reproduction. This

typical differentiation between the substratum and aerial growth, and the characteristic coloration of the colony and the aerial mycelium, which is of great importance as a basis for the separation of types, is had only under favorable conditions: in the presence of air and the proper medium. The colony is not smeary as in the case of most bacteria; the growth is solid and discrete. It can easily be lifted from the plate with a platinum needle, without breaking the colony. Placing the plate under the microscope, one can easily tell whether the organism is an actinomyces or bacterium, by the radiation of the thin hyphae from the colony into the medium. The surface of the growth is either smooth or undulated, ridged, folded, and even exfoliated, the colony having sometimes a lichenoid appearance. The edge is usually entire and filamentous. The substratum growth consists of very fine, branching mycelium, reminding one of bacterial structure, as there is no differentiation among wall, protoplasm, and cell sap. Septa are not formed and the younger branches are formed irregularly on the older ones. The aerial mycelium may cover only the central part of the colony, leaving a free margin, but very often the whole colony is covered with aerial mycelium. The latter usually consists of thicker hyphae than the substratum mycelium. They often end in club-like structures, or in spirals of different curvatures. Some of the species form spirals abundantly, the latter being generally characteristic of the type. The aerial hyphae break down into spherical, oval, or rod-shaped spores, of different sizes, and which are often united in pairs or in chains. Some organisms produce spores at an early stage, some only after they are several weeks old, some species do not form spores at all. The color of the aerial mycelium is characteristic of the species; sometimes it may be two-colored, at first white, only later developing the characteristic color. The color of the mycelium changes with the different media used, being influenced by the carbon and the nitrogen source. The colonies themselves are either colorless, or yellow, red, green, brown, black, blue, or of some other color. Many organisms produce a soluble pigment, which colors the medium, and this is also characteristic of the species. The three "violaceus" types produce a violet, blue or dark pigment on Czapeck's agar, while on potato only the "violaceus-ruber" produces the blue pigment, the other two species producing no pigment at all.

The growth on gelatin has been described before. Krainsky (13) found only the *Act. citreus* to produce an aerial mycelium on the bouillon gelatin; the present work has shown that many species produce an aerial mycelium on pure gelatin in distilled water, though not so readily as on Czapeck's agar.

Liquid media give characteristic growths with different species. All the organisms could be classified into four groups by their growth in

Czapeck's solution, as follows: those producing (1) a flaky growth on the bottom of the flask; (2) flaky growth all through the medium; (3) individual, well defined colonies all through the medium; (4) combined flaky growth on the bottom and colony formation through the medium. The character of colony formation may be specific. With some species the colonies are attached to the wall of the flask; others form colonies which always float free. Colonies may unite in masses on the surface of the liquid or at the bottom of the flask.

Dextrose broth (1 per cent) in fermentation tubes was inoculated with a considerable number of the species studied in order to determine their gas production. Not only was no gas produced in any case, but every organism failed to grow in the closed end of the tube. This fact shows that the actinomyces are not preferential anaerobes. This has already been pointed out by Beijerinck, who classed them as facultative anaerobes.

An interesting point in the growth of some actinomyces is the production of rings in the colony. This is especially prevalent on poor media. The ring formation may take place in the vegetative portion of the colony as well as in the aerial mycelium. So far no adequate explanation has been found for this phenomenon.

CLASSIFICATION OF THE ACTINOMYCES.

Most of the work on the identification of the actinomyces has been undertaken from the pathological point of view. Different media have been used and different characters recorded. The result is, that with few exceptions, it is impossible to identify soil organisms with those that have been described before. For example: "*Act. chromogenus*" was supposed to be a well defined organism, yet Krainsky (13) had four representatives of the group. The authors have eight "chromogenus" types, each with such well defined characters as to make it almost impossible to classify them as one species.

The only work that could be satisfactorily used for the classification of the organisms at hand was that of Krainsky (13). Sanfelice (21) has classified the actinomyces into three groups: (1) albus, (2) flavus, (3) violaceus. This grouping, based merely on the color of the colony, is purely arbitrary, because, as has been pointed out, the color varies with the media used. Krainsky classifies the actinomyces into: (1) the Macro-group, which appear early on the plates, form large colonies, and bear oval or spherical conidia, (2) the Micro-group, appearing late, in small colonies, and producing spherical conidia. They are strong cellulose decomposers. (In this group belong the organisms producing violet and yellow colonies.)

This method of grouping is far from perfect. The size of the colony

and the time of its appearance on the plate depend not only on the organism itself, but on the media used and the incubation temperature. Also, the fact that both groups may produce spherical conidia is confusing.

In the present work it was thought advisable to describe the organisms as to their characteristic growth on different media. When sufficient material has accumulated it may be possible to work out a system of classification having for its foundation the more stable and important characters of the organisms.

While the grouping of the organisms according to the rapidity with which they liquefy gelatin and according to their production or non-production of pigment in this medium, does not meet all the objections to such a classification, it is believed by the authors that it offers a starting point for identification. The term "rapid liquefaction" is here applied to liquefaction of 15 per cent gelatin in distilled water, in three days at 15° to 17° C.

Over one hundred organisms have been isolated from the soil. These represent 30 species described in the present article. Several organisms have produced such scanty and uncharacteristic growth on the media used that it has been thought advisable to keep them under observation for a longer period, in the hope of detecting important stages in their life history.

DESCRIPTION OF THE ACTINOMYCES ISOLATED FROM THE SOIL.

Act. violaceus, n. sp.

Czapeck's Agar. Colony at first colorless, turning red and blue, colors seen very clearly in the reverse. The red color is soon absorbed by the profuse production of a cyanine blue (Rdg.* ix-51-m) pigment, which diffuses through the medium. Colonies 1 to 3 mm. in diameter, showing rapid growth and formation of zones. Surface of colony smooth with a narrow, entire, white margin. Aerial mycelium appears at an early stage of the colony, at first white, then turning to mouse-gray (Rdg. li-15""). It has a silvery appearance due to the drops of water exuded upon the surface. Odor present, but weak. Microscopically two kinds of mycelium could be determined: the substratum mycelium consisting of fine closely branched filaments of a red to blue color; and the aerial growth consisting of thicker, straight filaments with very little branching at the edge of the colony, but more branching in the centre. Numerous spirals are found in the aerial mycelium. These as well as the hyphae break up readily into oval to rod-shaped conidia, 0.8 to 1.5 x 0.7 to 1.0μ.

Gelatin. Spreading, dense, colorless growth, with an early production

* This abbreviation throughout this paper refers to *Ridgeway*, "Color Standards and Nomenclature."

of a white aerial mycelium, underlaid by a pinkish coloration. Gelatin around the colony slowly liquefied, remaining clear.

Potato plug. After 36 to 48 hours, growth appears as a mass of well defined, round colonies, 1 mm. in diameter. White aerial mycelium is produced at an early date. Color of plug is at first unchanged, but after 4 to 5 days red and blue pigments are produced, either of them predominating; these also slightly color the white aerial mycelium.

Czapeck's solution. Growth consists of a flaky mass on the bottom of the flask, with numerous, small, round colonies all through the medium.

Glucose solution. A solid growth in the form of a grayish-white ring is formed on the surface, close to the side of the flask, with no growth through the medium. White to gray aerial mycelium appears at an early stage.

Hab. Isolated several times from the California adobe soil. Herbarium Nos. 8 and 44.

Act. violaceus-Caeseri, n. sp.

Czapeck's Agar. Growth very slow, consisting of gray colonies, 2 to 3 mm. in diameter. Surface of colony glossy and much folded. Aerial mycelium produced very late; it is pure white, with no shading into gray. A plum-purple (Rdg. xxiv-57-m) pigment is produced at an early stage, and gradually diffuses all through the medium. Pigment is much darker than that produced by *violaceus-ruber,* and no red tinge is ever observed. Medium becomes so dark from the diffused pigment as to be black by reflected light. Weak odor is given off. Microscopical examination shows the mycelium to consist of fine filaments, with the production of numerous open spirals in the aerial mycelium. Conidia could not be demonstrated.

Gelatin. Growth very small, with no aerial mycelium produced. Gelatin around the colony is rapidly liquefied, with no change in color.

Potato plug. Growth consisting of small yellowish colonies, which develop very slowly. Little aerial mycelium seen even after cultures are 20 days old. The color of the potato is not changed. No pigment is produced.

Czapeck's solution. Small flaky growth on the bottom of the flask. None through medium. All medium is colored blue at an early date. This is the only organism which colored deeply the Czapeck's solution.

Glucose solution. Thin, flaky growth on bottom of the flask, with none through medium.

Hab. Isolated once from the upland California soil. Herbarium No. 31.

Act. violaceus-niger, n. sp.

Czapeck's Agar. Colony at first dark gray, turning almost black. 2 to 4 mm. in diameter. Surface glossy, much folded with a very thin,

gray margin. A white to gray aerial mycelium is produced after the colony has well developed. A bluish black pigment is produced at a later stage of its growth. The pigment slowly dissolves in the medium, turning almost black. Odor fairly strong. Microscopically two types of mycelium were found: the thin, branching filaments of the substratum, and the thick filaments of the aerial mycelium. The aerial mycelium fragments not very rapidly, producing a few conidia, spherical and oval, 1.2 to 2.3 x 1.2 to 1.5μ. These often occur in chains.

Gelatin. Gray growth on gelatin, with no production of aerial mycelium. Gelatin around colony rapidly liquefied, but without any change in color.

Potato plug. Growth at first very slight, but after 48 hours it develops in a yellowish-gray continuous thick smear, which turns brown at a later date. White aerial mycelium covers the growth late. Plug is not discolored.

Czapeck's solution. Colonies large, 2 to 3 mm. indiameter, appearing at the bottom and surface of the solution, but none throughout the medium. The colonies are bluish in color, with a regular margin. Medium is not colored.

Hab. Isolated once from the upland California soil. Herbarium No. 39.

Act. erithrochromogenus, Krainsky.

Czapeck's Agar. Colonies small, round, 2 to 3 mm. in diameter, with a slightly cut margin. Color of colony is at first tawny-olive (Rdg. xxix-17"-i) to a buffy-brown (Rdg. xl-17"-i). Surface smooth at first, then lichnoid. White to gray aerial mycelium found at a later stage; this does not cover the whole surface, appearing only at separate parts of it. Reverse of colony dark. The production of soluble brown pigment at an early stage of its growth is characteristic of this organism as well as of all the other chromogenes species. The substratum mycelium consists of fine filaments with little branching. Aerial mycelium does not show any distinguishable structure. Conidia are abundant and fairly large, rod-shaped, 1.5 to 2.4 x 1.1 to 1.4μ. A strong odor can be easily detected.

Gelatin. After several days of growth, each colony is found at the bottom of a small pit. Centre of colony is yellow, edge hyaline. The radial mycelium extends into the unliquefied portion of the gelatin. Slight white aerial mycelium is found on the surface of the older colonies. Gelatin around the colony is liquefied very slowly and is colored brown.

Potato plug. Growth consisting of small individual colonies, forming a continuous streak all over the plug. Colonies are at first yellow-gray, becoming with age dirty-gray and glossy in appearance. Aerial mycelium not formed readily. Plug is darkened.

Czapeck's solution. Growth consists of individual, round, brown colonies 1. to 1.5 mm. in diameter all through liquid and on surface.

Glucose solution. Very little growth takes place.

Hab. Isolated from the upland and adobe California soils. Herbarium Nos. 17 and 18.

Act. diastato-chromogenus, Krainsky.

Czapeck's Agar. The description of this organism coincides very closely with that given by Krainsky for the organism under the same name. Colonies fairly large (3 to 5 mm.), at first colorless, then becoming brownish. Gray aerial mycelium colors all the colony at an early stage without leaving any free margin. Small drops of water are exuded upon the surface. Reverse of colony is brown, and a light brown pigment is produced dissolving through the medium. Odor present, but weak. Microscopically, the aerial mycelium is found to consist of long filaments, with very little branching. These break up readily into rod-shaped conidia, 1.5 to 1.8 x 1. to 1.2μ.

Gelatin. Gelatin is rapidly liquefied with the production of a brown color.

Potato plug. Growth light gray turning brownish, in one continuous streak, with several small separate colonies (1 mm.). White aerial mycelium appears at an early date and covers the whole growth. Potato is blackened.

Czapeck's solution. Flaky growth on the bottom of the liquid, and a pellicle on the surface. No growth through the medium.

Glucose solution. Flaky growth on the bottom of the liquid, and a ring of individual colonies 1 to 2 mm. in diameter on the surface of the liquid close to the glass of the flask.

Hab. Isolated twice from the California adobe soil. Herbarium Nos. 41 and 47.

Act. purpeo-chromogenus, n. sp.

Czapeck's Agar. Colonies small, 0.5 to 1.5 mm. in diameter, developing very slowly. They are brown in color with a brown to black reverse. Surface of colony is glossy, and raised in the centre. A brown soluble pigment is produced, which shows a distinct purple tinge, when viewed by transmitted light. Aerial mycelium is formed later. When culture is over 4 weeks old a brownish purple to black surface mycelium is formed. Margin of colony is waxy-yellow in color and lichnoid in appearance. No odor could be detected. Microscopically, no difference could be seen between the substratum and surface mycelium, a condition which suggested the question whether or not the latter could be called aerial mycelium. No conidia could be found. Substratum mycelium seems to break up into spherical non-staining bodies, .75 to 1.μ in diameter (oidia?).

Gelatin. Growth very slow. Liquefaction of the gelatin is slow with the production of a brownish pigment only at a late period.

Potato plug. No growth on potato in 2 to 3 days at 30° C. Only after 10 days the colonies were found to be very small, orange colored, grouping in a bead-like fashion. Colonies become dark brown with age. Potato slightly colored brown.

Czapeck's solution. Flaky growth on the bottom of the flask.

Hab. Isolated once from the California adobe soil. Herbarium No. 49.

Act. viridochromogenus (*?*), Krainsky.

Czapeck's Agar. Colony 2 to 8 mm. in diameter, at first yellowish-gray, slightly raised above the substratum. Surface glossy, granular, at first yellowish-brown and finally becoming dark green in color. White aerial mycelium appears first at the edge of the colony, rapidly advancing toward the centre, until finally the whole colony is covered with a white-gray aerial mycelium. Margin of colony is colorless, regular. Reverse of colony is colored at first yellowish-gray, later becoming dark brown. A dark brown to black pigment is produced which dissolves through the medium. Microscopically, the aerial mycelium is found to consist of a dense mass of filaments with little branching. The filaments fragment easily into long pieces, and these usually break up into oval-shaped spores, 1.2 to 2 x 0.75 to 1.1μ. Odor present but not very pronounced.

Gelatin. Gelatin rapidly liquefied with the production of a brown pigment.

Potato plug. Growth much folded, continuous streak, at first grayish yellow to green, but finally becoming dark green. White aerial mycelium is produced. Potato becomes black.

This organism seems to coincide with Krainsky's "viridochromogenus," though it differs from it in some details.

Hab. Isolated once from garden soil at a depth of 8 inches. Herbarium No. 61.

Act. chromogenus group.

Besides the four species of "chromogenus" previously described, several more organisms have been isolated, which show the characteristic features of the "chromogenus." These cannot be classified as one species, because they show distinct characters from one another. All of them are characterized by a colorless colony, changing later to different shades of brown. Surface mycelium is either absent or produced when culture is over 2 to 3 weeks old, and is of a brown to black color, hardly differentiated from the surface of the colony. Some organisms may produce small white tufts at a late stage. Reverse of colony is brown to black.

A brownish pigment diffuses through the substratum. The colonies are hard, a condition due probably to the production of quinone, which is characteristic of all the "chromogenus" species, as was pointed out by Beijerinck. Gelatin is liquefied at first, but the liquefaction does not advance far; the hardening up is also probably due to the quinone production. A brown pigment diffuses through the unliquefied portion of the gelatin. These cultures have shown the same general characteristics as the *Act. chromogenus* isolated from potato scab, a culture of which was borrowed from the plant pathology department of the New Jersey Agricultural Experiment Station.

STRAIN No. 1. Colonies 3 to 5 mm. in diameter. Surface smooth, dry, with lichnoid margin. Color yellow becoming overlaid by a dry, thin, black sheet, with the production of white aerial mycelium in centre of colony at a late stage. Tendency to grow in individual colonies, and not to form a solid streak. General outline of colony is yellow to black. Reverse dark brown to almost black, medium colored brown. Microscopically, aerial mycelium was found to be either lacking or very scarce. Surface mycelium consists of thick hyphae, but no clubs observed. Conidia few, oval to rod-shaped, 1.2 to 1.8 x 1.μ.

Hab. California upland soil. Herbarium Nos. 40 and 48.

STRAIN No. 2. Colonies 3 to 5 mm. in diameter, colorless at first, becoming later gray. Surface glossy, ridged, with folds radiating from the centre of the colony. White aerial mycelium appearing in small tufts at a late stage. This is easily observed on mannite lacking in nitrogen, and used for the study of nitrogen-fixing bacteria. Microscopically, aerial mycelium was found to be scant, with numerous dark staining granules in the hyphae. Small oval conidia fairly numerous, 1. x 0.6μ. Filaments show a club-shaped appearance at the end.

Hab. California upland soil. Herbarium Nos. 45, 53 and 54.

STRAIN No. 3. Colony is of a dirty gray color, with an intensive ring formation. Scant white aerial mycelium develops when culture is 6 to 8 weeks old. Microscopically, aerial mycelium can hardly be differentiated; it shows a granular structure, with the formation of clubs at the end of the filaments. Some filaments are greatly enlarged. No conidia were observed.

Hab. Isolated once from garden soil 20 inches deep. Herbarium No. 36.

STRAIN No. 4. Colonies large, 5 to 8 mm. in diameter, at first gray, then becoming brown in color. Surface glossy, at first smooth, later becoming slightly wrinkled. Snow-white aerial mycelium is produced. No conidia and no clubs could be observed under the microscope. Aerial mycelium was found to be very dense.

Hab. California loam. Herbarium No. 22.

Act. exfoliatus, n. sp.

Czapeck's Agar. Colonies round, 2 to 3 mm. in diameter, of a Dresden-brown color (Rdg. xv-17'-k), with a wide sterile margin. Colony has a tendency to crack, and surface growth to exfoliate and peel off. The margin of the streak culture is peeled off, leaving medium free. Many cracks are found in centre of growth. White aerial mycelium is produced at an early date. A blue pigment is produced in the colony, not soluble in the substratum, but seen clearly through the aerial mycelium. Reverse of colony is brown to black. Microscopically, the aerial mycelium is found to be thick, 1.5μ in diameter. Conidia oval, 1.2 to 1.8 x 1. to 1.5μ. Odor weak.

Gelatin. Colony develops in the bottom of a liquefied pit, showing a dense yellow mycelium in the centre; edge of colony is hyaline. Radial mycelium extends into the unliquefied portion of the gelatin. White aerial mycelium is produced. Gelatin around the colony is slowly liquefied, with no color produced.

Potato plug. Growth continuous, thick, somewhat folded, at first colorless to gray, later becoming yellow. No aerial mycelium produced. Potato not affected.

Czapeck's solution. Many minute individual colonies all through medium and heavy pellicle on surface of liquid.

Hab. Isolated several times from the adobe and upland soils. Herbarium Nos. 20, 50 and 51.

Act. diastaticus (?), Krainsky.

Czapeck's Agar. Colonies 2 to 4 mm. in diameter, gray, later becoming colored pale yellow. Many rings are formed by growth of colony. Aerial mycelium drab-gray (Rdg. xlvi-17""d), with small white tufts protruding in several places. Reverse of colony brown to black, with the deep brown mycelium penetrating deep into the substratum. This organism seems to answer Krainsky's description of the "diastaticus." However, no biochemical studies have been undertaken as yet to prove the identity of this organism. Microscopically, two kinds of aerial mycelium were found. The white mycelium is very dense, made up of straight, branching filaments. The brown aerial mycelium is made up of dense clusters of fine and narrow spirals. Oval conidia, 1.1 to 1.5 x 1. to 1.2μ. Odor weak.

Gelatin. Small colonies with white aerial mycelium produced at a late stage. Gelatin around the colony is rapidly liquefied, with no coloration.

Potato plug. Colonies small, 1 to 1.5 mm. in diameter, covering all the plug. Color of colonies white-gray with white aerial mycelium, which later becomes ash-gray. Each colony is pitted and raised 1 to 2 mm. above surface of plug. Potato darkened in two days.

Czapeck's solution. Flaky, colorless growth all through medium and on surface.

Glucose solution. Heavy gray ring of colonies on surface of liquid.

Hab. Isolated once from California sandy loam. Herbarium No. 13.

Act. albus, Krainsky.

This is a very common soil organism, isolated by Krainsky and reported by many investigators, as one of the few actinomyces found in the soil. The identification of this organism was based on Krainsky's description of the *Act. albus.*

Czapeck's Agar. Colonies 3 to 5 mm. in diameter, uniform in size, pale neutral-gray color (Rdg. liii-n. g.-d). White aerial mycelium produced early. Uniform growth on slant. Odor weak. Profuse spore formation. Conidia are both spherical and oval, 1.2 to 1.6 x 1.1 to 1.4μ.

Gelatin. White aerial mycelium develops readily on the surface of the colony. Gelatin is liquefied rapidly with the production of a brown coloration.

Potato plug. Thin gray to brownish streak covered with white aerial mycelium, which becomes gray with age. Potato is not colored.

Czapeck's solution. Growth consists of small, white, individual colonies, 1 to 2 mm. in diameter, on glass of flask. None through medium and on bottom.

Glucose solution. Small growth consisting of a white ring on surface close to glass of flask.

Hab. Isolated several times from the adobe soil and garden soil at a depth of 8 inches. Herbarium Nos. 6, 30 and 63.

Act. alboatrus, n. sp.

Czapeck's Agar. Colonies colorless and fairly large, 5 to 8 mm. in diameter. Surface of colony at first smooth, later becoming ridged, with folds radiating from the centre. White aerial mycelium covers the whole colony at an early date, leaving a narrow glossy margin, which has a lichnoid appearance. Small drops of water are exuded upon the surface. Reverse of colony is at first light brown, becoming with age dark reddish-brown. Strong odor is present. Microscopically, aerial mycelium consists of dense, closely branched filaments. These break up into long fragments, which have nothing in common with what is ordinarily understood as conidia. No true conidia observed.

Gelatin. Gelatin is rapidly liquefied, with no coloration.

Potato plug. Growth forms a thick, continuous streak, much folded, glossy, and of a white color. Potato does not change in color. A rose-colored aerial mycelium appears only after 20 days at 30° C.

Czapeck's solution. Flaky growth on the bottom of the liquid, with few small colonies on the surface.

Hab. Isolated once from the adobe soil. Herbarium No. 52.

Act. reticuli, n. sp.

Czapeck's Agar. Colony colorless, 3 to 5 mm. in diameter, becoming covered soon with a thin white cottony growth. This white aerial mycelium is characteristic of the organism. It is very fine, forming a woven net over the whole surface of the colony, with holes of about 0.5 mm. Reverse of colony creamy, later becoming brownish. Aerial mycelium abundant, consisting of filaments having no branches or very short ones. Spherical conidia are abundant, 1. to 1.4μ. Odor weak.

Gelatin. Rapid liquefaction takes place with the production of a soluble brown pigment only after 6 days.

Potato plug. Growth consists of brown, numerous colonies, 0.25 to 3 mm. in diameter, all over plug. Colonies are pitted, and covered with white aerial mycelium. Potato darkened.

Hab. Isolated from upland and adobe soils. Herbarium Nos. 43 and 95.

Act. citreus, Krainsky.

Czapeck's Agar. Colonies 3 to 5 mm. in diameter, with centre raised above substratum. Color of colony varying from olive-yellow (Rdg. xxx-23''') to citron-yellow (Rdg. xvi-23-b). Aerial mycelium of same color as colony, covering all colony without leaving any free margin. Reverse of colony deep colonial buff (Rdg. xxx-20''-b) to a deep yellow color, becoming with age yellow brown. Color of medium is unchanged. Odor weak. Microscopically, the substratum mycelium is found to consist of fine, branching filaments. Aerial mycelium consists of short, much branching, and tangled filaments. Conidia numerous, spherical to slightly oval, 1.2 to 1.8 x 1.2 to 1.5μ. They do not stain evenly, showing a clear centre.

Gelatin. Colonies very dense, yellowish, with white aerial mycelium. Gelatin around the colony is rapidly liquefied with no discoloration.

Potato plug. Growth appears as a gray to yellow thin smear, with white aerial mycelium. Color of potato unchanged.

Czapeck's solution. Slight flaky growth on the bottom of the flask, medium remaining clear.

Hab. Isolated several times from the orchard soil at depths of 1 inch and of 20 inches, and from the garden soil at a depth of 20 inches. Herbarium Nos. 29, 82 and 87.

Act. flavus, Krainsky.

Czapeck's Agar. Colonies 3 to 5 mm. in diameter, olive-yellow (Rdg. xxx-23'') in color, showing a characteristic ring formation. Growth on streak has a tendency to form individual colonies. Aerial mycelium light drab (Rdg. xlvi-17''''-C), with a wide sterile margin. Reverse of colony yellow to olive-yellow; no soluble pigment produced. The microscope

shows tangled aerial mycelium with little branching. The mycelium is swollen at intervals, forming the characteristic club shaped filaments up to 3μ in diameter. Spores oval and spherical, 0.9 to 2 x 0.9 to 1.1μ.

Gelatin. Growth dense, light yellow in color, with a shiny surface; no aerial mycelium is produced. Liquefaction of the gelatin around the colony starts early, but does not advance very rapidly. A light brown pigment is produced.

Potato plug. A finely wrinkled continuous growth gray to brown in color is produced in two days. White aerial mycelium develops only after 8 to 10 days' incubation at 30° C. Potato colored brown.

Czapeck's solution and Glucose solution. Flaky growth on bottom of the flask, none through medium.

Hab. Isolated from upland and adobe soils. Herbarium Nos. 23 and 38.

Act. parvus, Krainsky.

Czapeck's Agar. Colony 1 to 3 mm. in diameter, of a honey-yellow color (Rdg. xxx-19"). Surface of colony smooth and glossy, long remaining without any aerial mycelium. Scanty gray-yellow aerial mycelium appearing only late. Reverse of colony brownish. Substratum mycelium fine, very dense, and tangled. Aerial mycelium consisting of thicker branching filaments. Conidia oval, 1.2 to 1.8 x 0.9 to 1.3μ. No odor could be detected.

Gelatin. Colonies yellow, slowly developing on the gelatin. Liquefaction of the gelatin around the colony advances slowly, with no pigment production.

Potato plug. Growth continuous, folded, with a lichnoid margin, gray to brown in color. White aerial mycelium produced at an early stage. Potato colored black.

Hab. Isolated from garden soil at a depth of 12 inches, and Oregon soil. Herbarium No. 76.

Act. griseus, Krainsky.

Czapeck's Agar. Colony round, 3 to 6 mm. in diameter, growing rapidly with the formation of numerous rings. Color of colony olive-buff (Rdg. xl-21'"-d). Aerial mycelium appears at an early stage; it is a thick powdery mass of a water-green color (Rdg. xli-25'"-d). The color of the aerial mycelium is somewhat lighter than that described by Krainsky. Odor present, but weak. Microscopically, the aerial mycelium is found to consist of long filaments, with very little branching. These fragment readily into rod-shaped conidia, 1. to 1.5 x 0.8μ. The conidia occur often in chains; they do not stain readily in the centre; so that they produce a beaded effect.

Gelatin. Greenish-yellow colonies with a prominent substratum

growth. Aerial mycelium produced of a white-gray color. Liquefaction of the gelatin takes place rapidly with no pigment production.

Potato plug. Growth yellow, continuous, with the formation of individual colonies separated from the streak. Aerial mycelium at first white-gray, then changing into the characteristic water-green color. Potato is colored brown.

Czapeck's solution. Flaky growth on the bottom and throughout the medium.

Glucose solution. Heavy growth consisting of round colonies (1 to 3 mm. in diameter) floating on the surface and forming a ring in contact with the glass. A powdery white aerial mycelium soon covers the surface of the colonies. Some growth is found on the bottom of the liquid.

Hab. Isolated from California adobe soil. Herbarium Nos. 33 and 34.

Act. albo-flavus, n. sp.

Czapeck's Agar. Colonies small (2 to 4 mm.), round, colorless and glossy, at first, later becoming yellowish in color. Aerial mycelium forming a white powdery mass, with a yellow tinge developing later. Reverse of colony is yellowish, but medium is not colored. Under the microscope the aerial mycelium was found to have a tendency to produce special structures, consisting of a mass of hyphae massed together into a rope, and from this rope fine filaments coming out in the shape of side branches. The structure looks like the root of a tree and fine rootlets coming out on the side. This special structure persisted even in stained preparations. No conidia were observed. No spirals produced. Weak odor present.

Gelatin. Rapid liquefaction of the gelatin with no pigment production. The colonies are floating in the liquefied portion. No aerial mycelium produced.

Potato plug. Gray, continuous, thin growth. White aerial mycelium produced in 2 days at 30° C. Color of potato not changed.

Czapeck's solution. Few small colonies on the glass, none through medium or on bottom.

Glucose solution. White cylindrical colonies 3 x 1 mm., very uniform in size, growing together in a mass on the surface of the liquid. White aerial mycelium is produced at an early stage. The type of colony is very characteristic of this species, because it has not been observed in any other culture.

Hab. Isolated once from orchard soil at a depth of 20 inches. Herbarium No. 10.

Act. Verne, n. sp.

Czapeck's Agar. Colony 3 to 6 mm. in diameter, much folded, of an Isabella color (Rdg. xxx-19-i), with a wet surface. Margin of colony lichnoid. Aerial mycelium is little differentiated from the surface of the colony, and no such could be demonstrated. An elm-green (Rdg. xvii-27-

km) soluble pigment is produced, which diffuses rapidly all through the medium. Weak odor present. Microscopically, substratum mycelium found to be very fine, radial. Surface mycelium thicker and much branched. No segmentation or conidia could be demonstrated. Raised portion of old growth consists of a hard yellowish amorphous crust breaking into irregular fragments; there is no true aerial mycelium.

Gelatin. Globular colonies grow below the surface of the liquefied portion of the gelatin, varying in size from microscopic to 1.5 mm. No aerial mycelium is produced. Reverse of colonies is dark, due to the green pigment discoloring the medium. Gelatin is rapidly liquefied with no brown coloration.

Potato plug. Growth at first yellowish-gray, with a tendency to form individual colonies. Later it becomes thick and much folded. Scant white aerial mycelium is produced only on the tip of the growth. Color of potato is unchanged.

Czapeck's solution. Small colonies formed on the bottom of the liquid.

Glucose solution. Slight flaky growth on the bottom.

Hab. Isolated once from the upland soil. Herbarium No. 42.

Act. albosporeus, Krainsky.

Czapeck's Agar. Colony Acajou red (Rdg. xiii-l'-1), with an early production of white aerial mycelium. Reverse of colony orange-red. Growth on streak continuous, without any tendency to form individual colonies. Answers closely Krainsky's description. Substratum mycelium fine, radial, red colored. Aerial mycelium coarser, white, much branched. No spirals produced. Conidia very distinct, formed readily; spherical and oval shaped 1. to 1.8 x 0.8 to 1.2μ, often occurring in chains.

Gelatin. Colonies at first yellow in centre and hyaline at the margin; later they become red colored, remaining hyaline at the margin. Gray mycelium is produced in the centre of the colony. Gelatin is rapidly liquefied, with no pigment production.

Potato plug. Growth very slight, translucent, gray, becoming orange colored, with white aerial mycelium.

Czapeck's solution. Small flakes all through the medium and on the surface with the production of a soluble rose pigment.

Glucose solution. A pinkish ring is formed at the surface in contact with the glass; fair growth downward in the medium.

Hab. Isolated once from the upland soil. Herbarium No. 26.

Act. Bobili, n. sp.

Czapeck's Agar. Colonies small, 1.5 to 2 5 mm., folded, with a much cut lichnoid margin. Color at first coral red (Rdg. xiii-5'), becoming with age Acajou to Pompeian red (Rdg. xiii-l'-3'i), with a light colored margin. Reverse is light red. No true aerial mycelium is produced.

Odor strong, mouldy. Microscopically, the surface mycelium was found to be very fine and dense. No true aerial mycelium or spores could be demonstrated. Old surface growth consisting of a mass of degenerated mycelium.

Gelatin. Colonies dense, some having slight aerial growth. Each colony found at the bottom of a liquefied pit. Gelatin rapidly liquefied, at first colorless, then becoming colored brown. Under the microscope peculiar hyaline aerial formations were observed in the form of wedge, up to 10μ wide at the base, and tapering to a point. When these were smashed under the cover slip, nothing more could be found.

Potato plug. Growth gray to red, spreading all over plug. Surface of growth dry and much folded. Scant white aerial mycelium formed late at the tip of plug. Potato becoming brown with age.

Czapeck's solution. Colonies, 2 to 3 mm. in diameter, found all through liquid and on the bottom. Colonies are colorless at first, later the centre of the colonies becomes orange.

Glucose solution. Flaky growth on bottom, none on surface.

Hab. Isolated from adobe and garden soils. Herbarium Nos. 15 and 37.

Act. Californicus, n. sp.

Czapeck's Agar. Colonies small, 1 to 2 mm., round, vinaeceous colored (Rdg. xxvii-1"-d), growing deep into the substratum with almost no surface growth. The growth of the mycelium into the substratum can easily be followed by the red growth penetrating, 1 to 2 cm. deep, into the medium. No soluble pigment is produced, the color is found in the medium only where the mycelium has penetrated. Light neutral gray (Rdg. liii-n. g.-C) aerial mycelium covers surface in the form of a dry, powdery, thin layer. Microscopically, an abundant formation of open spirals could be found, 3.5 to 6μ in diameter. These break up easily into perfectly spherical conidia, which are very uniform and quite numerous, 1.2 to 1.5μ in diameter, often occurring in long chains. No odor could be detected.

Gelatin. Dense colorless colonies are abundant, with a gray-white aerial mycelium. Gelatin around the colony is slowly liquefied, with no pigment production.

Potato plug. Growth at first yellow to red, glossy, and shiny, with age becoming reddish-brown. No aerial mycelium produced. Potato not changed in color.

Czapeck's solution. Thin flaky growth all through medium and on surface.

Glucose solution. Pinkish ring formed at the surface, close to the glass. Scant flaky growth through medium.

Hab. Isolated once from the California sandy loam. Herbarium No. 3.

Act. Lipmanii, n. sp.

Czapeck's Agar. Colony 3 to 5 mm. in diameter, at first colorless, later becoming light brown. Centre of colony is elevated, giving it a conical appearance. There is an abundant production of aerial mycelium, which changes from neutral-gray (Rdg. liii. n. g.) to gray (Rdg. liii-6), with a white margin and white tufts all over surface. A ring formation takes place, alternating gray and white zones. Surface is smooth or slightly ridged. Minute drops of water exude upon the surface, giving the growth a silvery appearance. Reverse of colony is first light brown, then changes to almost black. No soluble pigment is produced. Under the microscope the aerial mycelium is found to be much branching and fragmenting readily into spherical or oval conidia. The conidia are 1. to 1.5 x 0.8 to 1.1μ, often occurring in chains. Odor weak.

Gelatin. Growth colorless, with white-gray aerial mycelium. Gelatin is rapidly liquefied with no pigment production. One strain of this organism (4) produced a cerro-green (Rdg. v-27-m) growth with white aerial mycelium.

Potato plug. Growth on the potato varies slightly with the different strains of the organism. It is usually a folded white to brownish smear, turning gray to gray-green. Aerial mycelium is white to ashy-gray. Color of the potato remains unchanged. The smear of one strain (12) is sulphur-yellow, of another (4) olive-green; otherwise the characters are alike.

Czapeck's solution. Flaky growth on bottom and surface, small white colonies all through the liquid.

Glucose solution. Grayish-white ring formed at the surface, close to glass.

Hab. This very common soil organism has been isolated by the writers many times from different soils, from the adobe, California sandy loam, and garden soil at a depth of 30 inches. Herbarium Nos. 4, 5, 7, 12, 58 and 62.

Act. Rutgersensis, n. sp.

Czapeck's Agar. Colony 3 to 8 mm. in diameter, slightly raised in the centre. Substratum mycelium penetrates deep into the medium. Surface colorless with an irregular margin. Aerial mycelium is produced at an early stage; it is at first gray, later becoming pale gull-gray (Rdg. liii-c. g). Zonation takes place in the formation of alternate gray and white rings. Reverse of colony changes from white to brownish. Microscopically, the aerial mycelium is found to consist of long, branching filaments, loose at the margin, but dense in the centre. There is an abundant spiral production of the close and open type. Conidia spherical and oval, 1. to 1.2μ in diameter, having a tendency to bi-polar staining. A strong odor is produced.

Gelatin. Rapid liquefaction of the gelatin takes place with no pigment production. No aerial mycelium formed.

Potato plug. Growth varies from a gray to a dark solid streak with scant formation of white aerial mycelium. Color of potato not changed.

Hab. This form, another common soil organism, has been isolated repeatedly by the writers from the local soils, from garden soil at depths of 1, 20 and 30 inches, orchard soil at 4 and 20 inches, timothy soil at 4 inches. Herbarium Nos. 67, 75, 79, 80, 83, 86 and 91.

Act. aureus, n. sp.

Czapeck's Agar. Colony 4 to 5 mm. in diameter, and translucent in color when 7 days old. Surface smooth. Aerial mycelium appears at an early date, at first mouse-gray (Rdg. li-15""), then changing into a cinnamon-drab color (Rdg. xlvi-13""). Ring formation takes place by the alternation of white and drab colored zones. Reverse at first white, changing to brown and almost dark brown. No soluble pigment produced. A characteristic exudation of dirty gray drops of water takes place in the centre of the colony, forming a small ring. Weak odor present. Aerial mycelium is characterized by the formation of numerous long spirals 17 to 20μ long, and 4 to 5μ in diameter. Spherical and oval conidia formed abundantly, 1. to 1.5 x 1. to 1.2μ. They stain readily, sometimes in a bi-polar manner.

Gelatin. Liquefaction starts rapidly in 3 to 4 days at 15° to 17° C., with no pigment production; then it becomes slower, with the production of a deep brown pigment in the unliquefied portion. White aerial mycelium is produced.

Potato plug. Growth continuous, folded, raised above the potato, of a gray to brown color. White to gray aerial mycelium appears early. Color of potato becomes black.

Hab. This forms also a common and numerous group of soil organisms. It has been isolated repeatedly from the local soils; garden soil at a depth of 1 inch, orchard soil at 12 and 30 inches, timothy soil at 4 inches, and Oregon soil. Herbarium Nos. 66, 68, 70, 71, 84 and 89.

Act. Halstedii, n. sp.

Czapeck's Agar. Colony gray, translucent, 4 to 8 mm. in diameter when 7 days old. Centre of colony is dark, with a large hyaline margin. Surface smooth. Aerial mycelium appears at an early date; it is at first white, then gull-gray (Rdg. liii-c. g.). Reverse of colony is colorless, turning dark in the centre. Medium not discolored. Odor fairly strong. Microscopically, the gray aerial mycelium was found to consist of long, slender, and spreading filaments. Close spirals, 7 to 10μ in diameter, are borne as branches of the filaments. Conidia are oval to rod-shaped, 1.2 to 1.8 x 1. to 1.2μ, often occurring in chains; they show only polar staining.

Gelatin. Rapid liquefaction with the production of a brown pigment in the unliquefied portion.

Potato plug. Growth solid, folded, greatly raised above the potato, gray to brown in color. White aerial mycelium covers only tip of growth. Color of potato is changed to black.

Czapeck's solution. Growth consists of 1 to 2 mm. colonies on side of vessel and bottom.

Hab. This is a common subsoil organism, isolated repeatedly from the deeper soil layers, but not from the surface soil. Garden soil at depths of 12, 20 and 30 inches; orchard soil at 12, 20 and 30 inches. Herbarium Nos. 33, 56, 72, 77, 85.

Act. Fradii, n. sp.

Czapeck's Agar. Colony 2 to 4 mm. in diameter, colorless, thin, with a smooth surface. Aerial mycelium is produced early; it is a thick cottony mass of a sea-shell pink color (Rdg. xiv-11'-f), with white tufts of mycelium in many places. Reverse colorless. No soluble pigment produced. Odor weak. Aerial mycelium consists of thick, long, unbranched filaments, which become branched only when old. Conidia numerous, rod-shaped, .75 to 1.25 x 0.5μ, of a sea-shell pink color.

Gelatin. Rapid liquefaction with no color production. White aerial mycelium is produced early.

Potato plug. Growth glossy, thick, of a zinc-orange color (Rdg. xv-13'). White to rose aerial mycelium. Color of plug not changed.

Czapeck's solution. Numerous minute colonies all through medium and on surface.

Hab. Isolated once from the adobe soil. Herbarium No. 55.

Act. roseus, Krainsky.

Czapeck's Agar. Colony 2 to 3 mm. in diameter, of a pale brownish vinaceous color (Rdg. xxxix-5'''-f). Growth of colony is limited. Aerial mycelium is of the same color as the colony, and is produced at an early date. Medium uncolored. No odor or odor very weak. The general characters of the organism coincide closely with those given by Krainsky. Microscopically, the species is characterized by the formation of numerous close spirals. Conidia formation takes place early; they are abundant and oval in shape, 1.5 to 2 x 1. to 1.2μ.

Gelatin. Slow liquefaction of the gelatin with the production of a deep brown pigment which spreads rapidly through the unliquefied portion of the gelatin.

Potato plug. Gray-yellow continuous streak on the plug. White aerial mycelium covers only tip of growth. Color of potato turns brown.

Czapeck's solution. Heavy, flaky mass all through liquid and on surface.

Glucose solution. Pink ring on the surface close to the glass. Some growth on the bottom.

Hab. Isolated from garden soil at depths of 8 and 12 inches. Herbarium Nos. 27 and 73.

Act. lavendulae, n. sp.

Czapeck's Agar. Colony 3 to 4 mm. in diameter, colorless, growing deeply into the medium in the form of long, colorless strands, with very little surface growth. Aerial mycelium appearing early in centre of colony; it is deep vinaceous lavender (Rdg. xliv-65'''-d), with a large sterile margin. A strong odor is present. Microscopically, close spirals were found, 5 to 8μ in diameter. Conidia abundant, oval, 1.6 to 2. x 1. to 1.2μ.

Gelatin. Slow liquefaction with the production of a brown pigment only after 6 days.

Potato plug. Golden brown, wide, thin, continuous growth. Color of potato black.

Hab. Isolated once from orchard soil at a depth of 4 inches. Herbarium No. 69.

Act. purpurogenus, n. sp.

Czapeck's Agar. Colony gray-translucent, 3 to 6 mm. in diameter, radially much wrinkled. Centre of colony is elevated, and colony itself becomes lichnoid in appearance. Centre is covered by white to purplish aerial mycelium, shading into dark grayish lavender (Rdg. xliii-57'''-C). Reverse of colony brownish to dark brown. No soluble pigment produced. Odor weak. Aerial mycelium is found under the microscope to be dense and twisted. Conidia oval, 1. to 1.5 x 0.8 to 1.μ.

Gelatin. Slow liquefaction with purplish coloration. White aerial mycelium is produced at an early date.

Potato plug. Gray-dark, much folded, continuous growth, with a pearly lustre. It looks as if many small individual colonies were massed together to form the streak. White aerial mycelum appearing late. Color of potato turned black.

Czapeck's solution. Large, thin, radiating colonies, rarely through medium and on surface.

Hob. Isolated repeatedly from the garden soil at depths of 20 and 30 inches, and orchard soil at 20 inches. Herbarium Nos. 59, 65 and 90.

Besides the classified organisms, several more cultures of unidentified species are at hand. Most of these develop very slowly, and not enough data could be collected for any grouping. They are kept for further study.

TABLE IV.

TABULAR STATEMENT OF SALIENT FEATURES OF ACTINOMYCES.

Name of Organism	Growth on Czapeck's Agar				Gelatin (15% in dist. H_2O)			Liquid Czapeck	Dextrose Broth 1%	Potato Plug			Odor
	Color of Colony	Aerial Mycelium	Reverse	Medium Colored	Lique-faction	Color of Medium	Aerial Mycelium			Color of Colony	Aerial Mycelium	Plug Colored	
Act. violaceus-ruber	red and blue	gray	red and blue	blue	slow	none	present white	flaky	surface	gray	white	blue	weak
Act. violaceus-Caeseri	gray	white	blue	plum-purple	rapid	none	none	flaky	bottom flaky	yellowish	scant, white	not colored	weak
Act. violaceus-niger	gray-black	white	black	black	rapid	none	none	colonies	.....	brown	white	black	medium
Act. erithro-chromogenus	brown	white	brown	brown	slow	brown	present white	colonies	scant growth	yellow-gray	none	black	strong
Act. diastato-chromogenus ...	gray	white-gray	brown	brown	rapid	brown	none	flaky	flaky	gray	white	black	medium
Act. purpeo-chromogenus ...	brown	purple black	purple black	brown	slow	brown	none	flaky	.....	orange brown	none	not colored	none
Act. virido-chromogenus	green-brown	white	black	black	rapid	brown	none	.....	.....	green	white	black	medium
Act. chromogenus (group)...	brown	white	black	brown	rapid	brown	none	.....	.....	brown	white	black	medium
Act. exfoliatus	brown	white	black	not colored	slow	none	present	colonies	.....	gray-yellow	none	not colored	very weak
Act. diastaticus	gray	drab gray	dark	not colored	rapid	none	present	flaky	ring	gray-white	white	dark	weak
Act. albus	gray	white gray	gray	not colored	rapid	brown	present	colonies	ring	brown	white-gray	not colored	weak
Act. alboatrus	colorless	white	brown	not colored	rapid	none	none	flaky & colonies	.....	white	rosy	not colored	strong
Act. reticuli	colorless	white gray	creamy	not colored	rapid	brown	none	.....	.....	brown	white	dark	weak
Act. citreus	olive yellow	white and yellow	gray	not colored	rapid	none	white	flaky	little growth	gray	white & yellow	not colored	weak
Act. flavus	olive yellow	light drab	yellow to olive-yel.	not colored	slow	brown	none	flaky	flaky	black	white	not colored	none

TABLE IV—(Continued).

TABULAR STATEMENT OF SALIENT FEATURES OF ACTINOMYCES.

Name of Organism	Growth on Czapeck's Agar				Gelatin (15% in dist. H_2O)			Liquid Czapeck	Dextrose Broth 1%	Potato Plug			Odor
	Color of Colony	Aerial Mycelium	Reverse	Medium Colored	Lique-faction	Color of Medium	Aerial Mycelium			Color of Colony	Aerial Mycelium	Plug Colored	
Act. parvus	yellow	yellow	brown	not colored	slow	none	none	.....	.....	gray	white	black	none
Act. griseus	olive buff	water-green	brownish	not colored	rapid	none	present	flaky	colonies	yellow & gray	white-gray	brownish	weak
Act. alboflavus	yellowish	white	yellowish	not colored	rapid	none	none	colonies	cylindrical colonies	gray	white	not colored	weak
Act. Verne	Isabella color	yellow-brown	dark	green	rapid	none	none	colonies	flaky	yellow gray	scant, white	not colored	weak
Act. albosporeus	Acajou red	white	red	not colored	rapid	none	present	flaky	flaky	orange	white	not colored	none
Act. Bobili	red	none	red	not colored	rapid	brown (late)	none	colonies	flaky	red	none	not colored	strong
Act. Californicus	red	gray	red	not colored	slow	none	present	flaky	flaky	red	none	not colored	none
Act. Lipmanii	colorless	gray	dark	not colored	rapid	none	white-gray	flaky & colonies	ring	.....	.....	.....	weak
Act. Rutgersensis	colorless	gray	white	not colored	rapid	none	none	.....	.....	gray	white	not colored	strong
Act. Halsteadii	gray to dark	white gray	colorless	not colored	rapid	brown	present white	.....	.....	brown	white	black	strong
Act. aureus	colorless	drab	white to brownish	not colored	rapid	brown	present white	.....	.....	brown	white	black	weak
Act. Fradii	colorless	sea-shell pink	creamy	not colored	rapid	none	white	colonies	.....	orange	rose	not colored	weak
Act. roseus	rose	rose	light rosy	not colored	rapid	brown	present	flaky	flaky	gray	white	brown	none
Act. lavendulae	colorless	lavender	creamy	not colored	slow	brown	none	.....	.....	brown	none	black	strong
Act. purpurogenus	gray	gray to lavender	brown	brownish	slow	purplish	white	.....	.....	dark	white	black	weak

KEY TO THE IDENTIFICATION OF THE ACTINOMYCES.

A. Gelatin liquefied rapidly, with no pigment produced in the unliquefied portion:

I. Spirals produced in the aerial mycelium:

1. No pigment produced in the substratum, *Act. Rutgersensis*
2. Pigment produced in the substratum:
   (a) Pigment dark blue, *Act. violaceus Caeseri.*
   (b) Pigment brown, *Act. diastaticus.*

II. No spirals produced in the aerial mycelium:

1. No pigment produced in the substratum:
   (a) Colony orange-red, aerial mycelium white, *Act. albosporeus.*
   (b) Colony rose-colored, aerial mycelium rosy, *Act. Fradii.*
   (c) Colony a mixture of white and yellow:
      (c') No conidia observed, *Act. albo-flavus.*
      (c'') Conidia present in abundance:
         x Conidia rod-shaped, colony powdery, gray-yellow. *Act. griseus.*
         y Conidia spherical and oval, colony compact, citron-yellow. *Act. citreus.*
   (d) Colony at first colorless, then becoming brown, to almost black:
      (d') Aerial mycelium white, no conidia observed, *Act. albotratus.*
      (d'') Aerial mycelium dark gray; conidia abundant, oval, *Act. Lipmanii.*
2. Pigment produced in substratum:
   (a) Color of substratum green, *Act. Verne.*
   (b) Color of substratum dark blue, *Act. violaceus-niger.*

B. Gelatin liquefied rapidly with the production of a brown pigment in the unliquefied portion:

I. Spirals produced in the aerial mycelium:

1. Colony rose-colored, with rosy aerial mycelium, *Act. roseus.*
2. Colony colorless, with golden brown aerial mycelium, *Act, aureus.*
3. Colony slightly brown, with white aerial mycelium, *Act. Halstedii.*

II. Spirals not produced in the aerial mycelium:

1. No pigment produced on the agar substratum:
   (a) Colony red to red-orange, with no aerial mycelium, *Act. Bobili.*
   (b) Colony white, with white aerial mycelium:
      (b') Aerial mycelium thin, rare, net-like, *Act. reticuli.*
      (b'') Aerial mycelium thick, white to gray, *Act. albus.*
2. Brown pigment produced in the agar:
   (a) White aerial mycelium produced early and abundant, *Act. diastato-chromogenus.*
   (b) White aerial mycelium not produced at all, or very late, *Act. chromogenus group.*
   (c) Surface of colony green, white aerial mycelium produced early, *Act. virido-chromogenus.*

C. Gelatin slowly liquefied, with no pigment production:
  I. Spirals produced in the aerial mycelium:
    1. Production of soluble red and blue pigments, *Act. violaceus-ruber.*
    2. No soluble pigment produced; red mycelium grows deep into the substratum, *Act. Californicus.*
  II. No spirals produced in the aerial mycelium:
    1. No pigment produced in the substratum, colony yellow. *Act. parvus.*
    2. Brown pigment produced, colony tends to crack, *Act. exfoliatus.*

D. Gelatin slowly liquefied, with the production of a brown pigment:
  I. Spirals produced: aerial mycelium lavender color, *Act. lavendulae.*
  II. No spirals produced in the aerial mycelium:
    1. Colony yellow, aerial mycelium gray, *Act. flavus.*
    2. Colony colorless, aerial mycelium white-purplish, *Act. purpurogenus.*
    3. Colony black, lichnoid, aerial mycelium scant, *Act. erithrochromogenus.*
    4. Colony purple, no aerial mycelium, *Act. purpeochromogenus.*

PHYSIOLOGY OF THE ACTINOMYCES.

Investigators find that most of these organisms grow best at 30° C. The minimum lies near 15° C. and the maximum is about 50° C.

The cellulose destroying power of the actinomyces has been studied by Krainsky (13), Scales (22), and several others.

As to their ability to liberate ammonia, Lutman and Cunningham (15) by inoculating nutrient broth with *Act. chromogenus* found 10 to 20 mg. of NH_3 in 50 c.c. of culture for from 14 to 30 days. Since liberation of ammonia from organic compounds is one of the most important factors in the study of organisms from the soil fertility standpoint, a series of experiments were started for the purpose of demonstrating the part played by the actinomyces in this process.

One hundred grams of soil and 2.42 gm. of cottonseed meal containing 155 mg. of nitrogen were placed in Erlenmeyer flasks and well mixed. Twenty cubic centimeters of water (equal to 70 per cent saturation for the soil used) were added. Two sets of flasks received 10 c.c. each and another two 40 c.c. each. Flasks were plugged and sterilized in the autoclave, 15 minutes at 15 pounds pressure. Cultures of the different organisms grown on Czapeck's solution were used for inoculation, 1 c.c. being added. Duplicates were used throughout. After 7, 14 and 30 days, respectively, the ammonia of the various sets was distilled off with MgO in the usual way. Table IV shows the results of the investigation.

One can readily see from Table V that the actinomyces do not play any appreciable rôle in the soil as ammonifyers, as judged by ammonification in soil sterilized by steam under pressure. Only six organisms gave more than 1 mg. of nitrogen as NH_3, all the rest accumulating less than that amount in 14 days. When the incubation period was extended to 30 days somewhat larger quantities of NH_3 were found to have been

TABLE V.

AMMONIA ACCUMULATION BY ACTINOMYCES IN THE SOIL.

Name of Organism	Period of Incubation	Moisture Content	Mg. of N. as Ammonia	Mg. of N. Average	Mg. of N. Minus check
Act. violaceons-ruber	14 days	20%	4.06 4.36	4.21	0.51
Act. violaceons-Caeseri	14 days	20%	3.64 4.10	3.87	0.17
Act. roseus	14 days	20%	3.52 3.06	3.29	—0.41
Act. erithrochromogenus	14 days	20%	4.06 4.67	4.37	0.67
Act. griseus	14 days	20%	4.71 4.10	4.41	0.71
Act. griseus	14 days	10%	3.33 3.46	3.40	—0.30
Act. griseus	14 days	40%	7.01 7.16	7.09	3.39
Act. griseus	30 days	20%	9.18 9.84	9.51	5.81
Act. albus	7 days	20%	4.56 4.12	4.34	0.74
Act. albus	14 days	20%	4.24 4.88	4.56	0.86
Act. albus	14 days	10%	3.16 3.94	3.55	—0.15
Act. albus	14 days	40%	7.01 7.63	7.32	3.62
Act. albus	30 days	20%	19.2 19.8	19.5	15.8
Act. citreus	14 days	20%	3.80 3.80	3.80	0.10
Act. flavus	14 days	20%	3.75 4.06	3.91	0.21
Act. albosporeus	14 days	20%	4.56 4.25	4.41	0.71
Act. albo-flavus	14 days	20%	3.75 3.75	3.75	0.05
Act. Lipmanii	14 days	20%	4.36 4.88	4.62	0.92
Act. Chromogenus (No. 22).	14 days	20%	4.51 3.75	4.13	0.43

accumulated. When the different moisture contents employed are compared it is found that the highest moisture content gave the highest ammonia accumulation.

It has been found that the actinomyces readily assimilate NO_2, NO_3, NH_3, and organic compounds of nitrogen. The characteristic point is that they reduce nitrate to nitrite, but not to free nitrogen or ammonia.

Not all actinomyces produce an odor, but as all representatives of the *Act. chromogenus* group seem to have an odor, it was formerly believed that this property was characteristic of the whole genus. The odor, when present, varies from very strong to very weak. The stronger odors are suggestive of a musty straw stack, and some of the milder ones of the smell of soil.

Heinze (11) suggested that the actinomyces probably play an important part in the formation of humus out of the organic matter of the soil, even when the soil has an acid reaction. Fousek (18) found that "streptothrix" do not nitrify; on the contrary, they reduce nitrates to nitrites, but without loss of free nitrogen. In view of the ready assimilation of nitrates, ammonia compounds, urea, and uric acid, by these organisms, it seems possible that they may help to "fix" nitrogenous fertilizers in the soil and prevent their being lost by leaching or denitrification. He (Fousek) states that the "streptothrix" have a favorable influence upon plant growth, because through their rapid decomposition of the organic matter, plant nutrients are set free and made available for the higher plants.

The knowledge that the actinomyces are strong cellulose decomposers and weak producers of ammonia leads one to think that the probable rôle of the organism in the fertility of the soil lies in the formation of humus. Organic matter when applied to the soil has to undergo a series of processes before it can be utilized by the higher plants. Among the most important of these is the decomposition of cellulose, and subsequent formation of humus. In this process the actinomyces probably play an important, if not a dominant part, together with the cellulose decomposing bacteria and fungi.

In arid soils where cellulose destruction has been found to be extremely rapid, we should, therefore, expect to find actinomyces in abundance. That this might be the case is indicated by the figures in Table III, representing the soils from southern California.

ACKNOWLEDGEMENT.

The writers wish to express their sincere thanks to Dr. J. G. Lipman for his valuable suggestions in regard to this work, and to Dr. J. W. Shive for his assistance in preparing the plates.

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PLATE I.

Fig. 1—Act. violaceus-ruber.
Fig. 2—Act. violaceus-Caeseri.
Fig. 3—Act. chromogenus, strain 22.
Fig. 4—Act. virido-chromogenus.
Fig. 5—Act. diastato-chromogenus.
Fig. 6—Act. erithro-chromogenus.
Fig. 7—Act. purpeo-chromogenus.
Fig. 8—Act. chromogenus, strain 40.

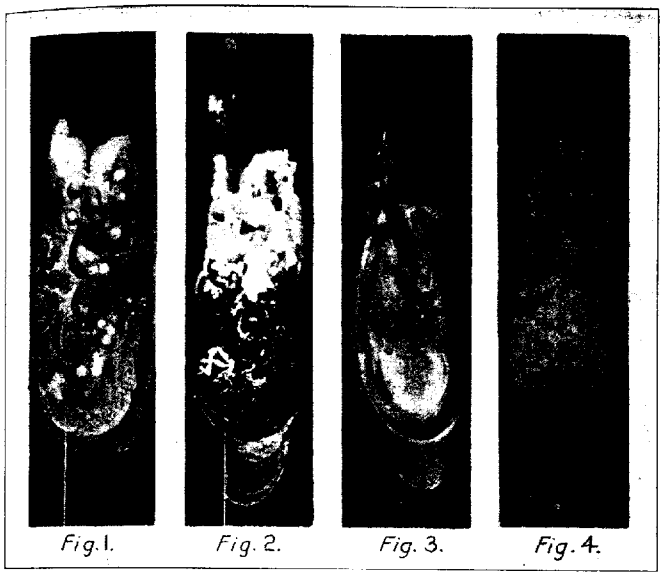

Fig. 1. Fig. 2. Fig. 3. Fig. 4.

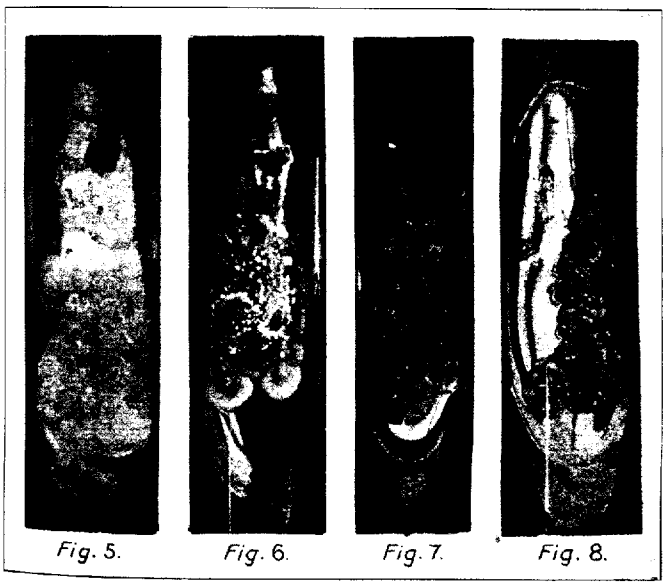

Fig. 5. Fig. 6. Fig. 7. Fig. 8.

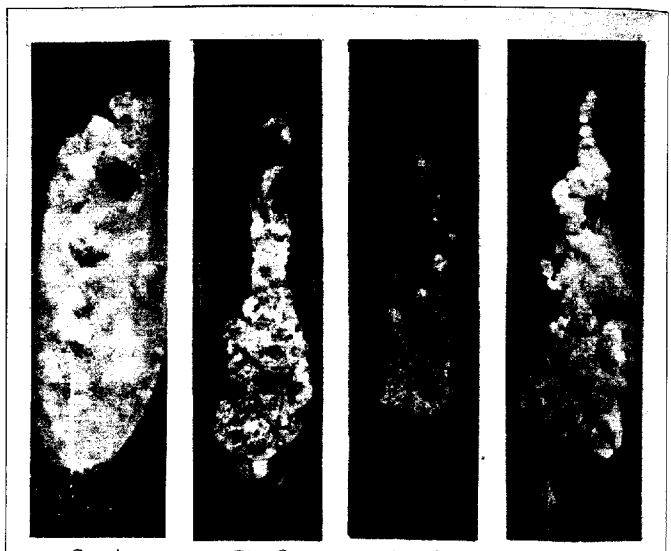

Fig. 1. Fig. 2. Fig. 3. Fig. 4.

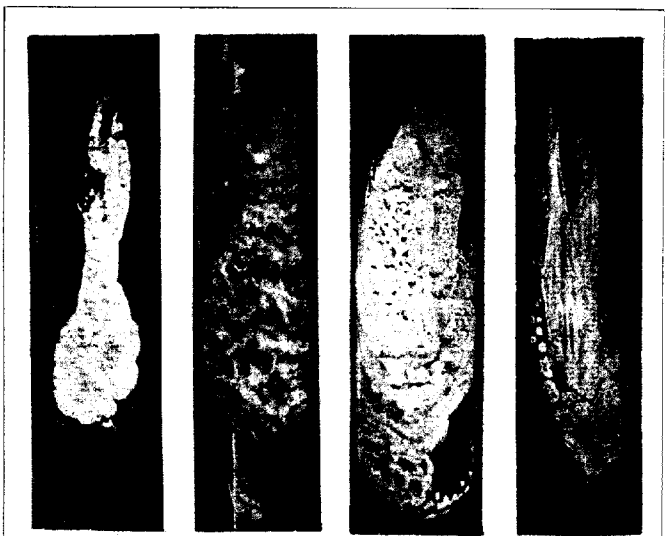

Fig. 5. Fig. 6. Fig. 7. Fig. 8.

PLATE II.

Fig. 1—Act. albus.
Fig. 2—Act. albotratus.
Fig. 3—Act. reticuli.
Fig. 4—Act. alboflavus.
Fig. 5—Act. albosporeus.
Fig. 6—Act. Verne.
Fig. 7—Act. griseus.
Fig. 8—Act. Californicus.

PLATE III.

Fig. 1—Act. citreus.
Fig. 2—Act. Bobili.
Fig. 3—Act. purpurogenus.
Fig. 4—Act. Lipmanii.
Fig. 5—Act. diastaticus.
Fig. 6—Act. Fradii.
Fig. 7—Act. exfoliatus.
Fig. 8—Act. flavus.
Fig. 9—Act. Rutgersensis.
Fig. 10—Act. roseus.

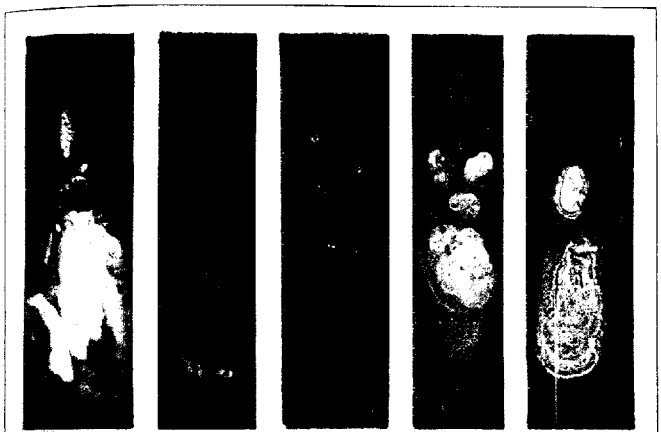

Fig. 1. Fig. 2. Fig. 3. Fig. 4. Fig. 5.

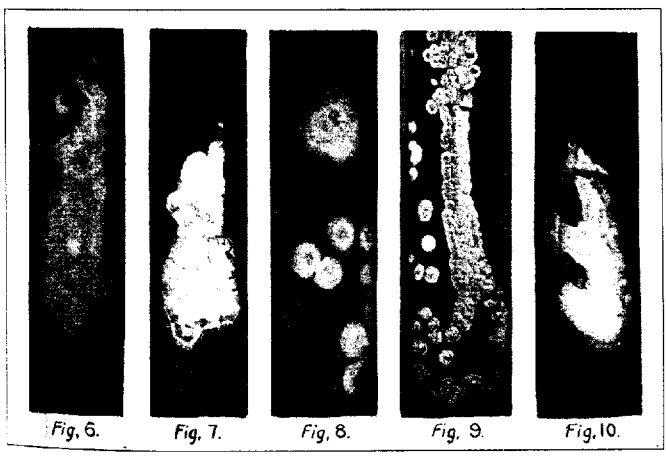

Fig. 6. Fig. 7. Fig. 8. Fig. 9. Fig. 10.

STUDIES ON SOIL PROTOZOA.

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The question of the activities of protozoa in the soil raised by Russell and his associates has turned the attention of the workers in soil microbiology to this group of microörganisms as a possible factor in soil fertility. The literature dealing with soil protozoa is given in several of the latest investigations, and the writer will call attention only to those which have a direct bearing upon the work at hand. The following lines are taken up in the present investigation, a preliminary report of which is given in the following pages:

1. Active protozoan fauna in the soil.
2. Numbers and types of protozoa in different soils at different depths, as shown by cultural methods.
3. Effect of protozoa on bacterial numbers and their decomposition of organic matter in the soil.

Activity of Protozoa in the Soil.

Many investigators, from Ehrenberg (4) and Rosenberg Lipinsky (13) down to the present time, have tried to find out whether the protozoa are present in the soil in a trophic condition, or are encysted temporarily in the soil. The latest investigations seem to contradict one another as to the very fact of the occurrence of living protozoa in soils under normal conditions of temperature and moisture. Goodey (6) maintains that ciliated protozoa exist in the soil only in an encysted condition. Where free water was to be found in the soil, the presence of active protozoa could be demonstrated. However, he worked only with the ciliates, which, as large organisms, are not likely to be found active in soils having comparatively low moisture contents. Francé (5) states that from March to October the protozoa are active if other conditions are favorable; after this period they encyst because of the frost or aridity. Martin (10) found that smaller amoebae and flagellates play the most important rôle in the phenomenon of sick soils, and that the most common limiting factor affecting the activity of protozoa in the soil is the average quantity of water. Only recently Martin and Lewin (11) have shown that the dominant active fauna of the soil, as shown by the fresh films, consists mostly of amoebae, thecamoebae and small flagellates. Koch (9), who has probably done the most recent work on the occurrence of living protozoa in the soil, concluded that living protozoa do not seem to be present in field soils with an unusual moisture content. He made his observations by placing several drops of sterile water on a clean slide, and stirring into the water by means

Received for publication November 15, 1915.

of a stirring rod a portion of soil to be examined. The examinations were made under the low power and for a period of not more than two minutes. There was very little chance for the observer to find the small flagellates among the soil particles. The motility of the protozoa is much slower in the soil than in culture solution, and it would be difficult to detect other than ciliates with the low power in such a short period.

To be able to get an idea of the time that is actually required for the protozoa to come out of encystment, the writer placed some soil in a deep cavity of a slide; the soil was covered with sterile tap water and examinations were made every five minutes for the detection of living forms of protozoa. After many repeated trials no flagellates could be found in a living condition before sixteen minutes, during which period the soil was covered with water. The first living ciliate was found only after the soil had been standing in contact with the water for 62 minutes. This led to the conclusion that there is no danger in extending the period of examination for active forms of protozoa to from 5 to 6 minutes. This length of time allowed closer observation among the soil particles, and a more thorough examination of the field. The high power (4 mm.) was used in case of doubt whether the organism was a small flagellate or a motile bacterium.

The error made in limiting the time of examination to only two minutes is made clear by the fact that in the 6 out of 20 greenhouse soils where Koch found protozoa, the organisms recorded are in five cases ciliates and amoebae, and only in one case flagellates. As was shown by Sherman (16), Cunningham (3), by the writer, and others, the number of flagellates is much larger than the number of ciliates, and we would expect that under conditions where moisture is the limiting factor, the smaller organisms would be able to lead an active life at a lower moisture content than the larger ones. Koch has also indicated that the moisture content of the soil is a primary limiting factor in the activity of the protozoa, while the texture and organic matter are secondary ones. It appears somewhat inconsistent, then, when one notes that he did not find any active protozoa at 34 and 36 per cent of moisture but did find them at 22.5 and 25.6 per cent in his examinations of greenhouse soils.

There is no doubt that there is a minimum moisture content for each soil below which protozoa cannot be found in a living condition. There must be another factor which is of greater importance than moisture, since this latter factor does not hold true with all the soils. Martin and Lewin (6), in addition to suggesting the use of fresh fixed films which bring out the active fauna of the soil, also used the method of adding water to the soil. In their examinations they were able to detect living organisms in different soils, but these were limited to small flagellates, amoebae and thecamoebae.

For this work the following soils were selected: (1) Sassafras loam rich in humus, and recently used for garden crops; (2) Sassafras loam poor in humus, in orchard; (3) Alloway clay, fairly rich in humus, in timothy; (4) Penn loam, in a variety of crops. Examinations were made every day for ten days in succession, during which period several rains occurred, some of which lasted a whole day. The moisture content was determined by air-drying 100 gm. of the soil. Several examinations were made with the low power (16 mm.) for a period of from 5 to 6 minutes. The high power was used to confirm the results in case of doubt.

The results of the examinations are set forth in Table I.

TABLE I.

NUMBERS OF ACTIVE PROTOZOA IN THE SOIL.

Days of Examination	July 15, 1915 1st day		2d day		3d day		4th day		5th day		6th day		7th day		8th day		9th day		10th day	
Soil	M.	Pr.	M.	Pr.	M.	Pr.	M.	Pr.	M.	Pr.	M.	Pr.	M.	Pr.	M.	Pr.	M.	Pr.	M.	Pr.
Humus	9.	....	9.5	....	13.5	S.F.	12.5	S.F.	13.	S.F.	10.	....	9.5	....	9.5	....	11.	....	14.	S.F.
Orchard	10.	....	9.5	....	12.	....	12.5	....	12.5	S.F.	9.	....	9.	....	9.	....	10.5	....	11.5	....
Timothy	14.	....	14.2	....	15.8	....	15.5	....	15.	....	14.	....	14.	....	13.5	....	15.	....	15.5	....
Penn Lm.	18.	....	18.	....	18.6	....	19.	....	20.	....	19.	....	18.	....	18.5	....	19.	....	20.	....

M = moisture in per cent. Pr. = Protozoa. S. F. = Small flagellates.

Glancing through the table one can readily see that the moisture content seems to be a limiting factor in the activity of protozoa in the soil. Small flagellates were found in the case of the rich humus soil at 13 to 14 per cent of moisture, which is the physical optimum for that soil. But no protozoa were found in the other soils except in one case, namely, in the orchard soil, where small flagellates were detected on the fifth examination. No protozoa were found by this method of examination to be active in the two heavy soils, even at a moisture content of 15.8 to 20 per cent. This tends to show that the moisture content of the soil is not the only limiting factor of the activity of protozoa, but that the structure and humus content of the soil likewise play an important part. Of the first two soils which are of the same physical texture and lie close to one another, the one rich in humus has shown greater protozoan activity than the other. Comparing the first two and the last two soils, we see that the lighter soils have shown some protozoan activity, while with the heavier soils no activity could be detected under correspondingly similar conditions of moisture by the same methods of examination. The protozoa found in the soils were all flagellates; had the moisture content of the soils been higher, no doubt ciliates also would have been found, because those soils develop ciliates in the culture solution, as will be seen later. As to amoebae and thecamaebae, it is thought that the method of examination was probably unsatisfactory for the detection of those organisms, if there were any living.

To learn something about the other factors which may play a part in making the protozoa active in the soil, the following experiment was conducted. Three soils of different texture were used, which will be termed "sandy soil," "loam soil," and "clay soil." Fifty-gram portions were placed in Erlenmeyer flasks and plugged with cotton. To one-third of the flasks organic matter was added in the form of dried blood in 3 gm. portions; thus an added excess of organic matter would probably elimmate any variations in the organic content of these soils. Another third of the flasks were sterilized after the proper amount of moisture had been added. The physical optimum moisture content was determined for each soil, and sterile water added in portions amounting to one-half, one, one and one-half, and two times the optimum for each soil. To those portions which had been sterilized, cultures of protozoa were added. (These were five-day-old cultures made by inoculating sterile Lohnis' soil extract with soil.) All flasks were kept in the incubator at 25° C. Examinations were made after three, five, eight and twenty days respectively. The occurrence of protozoa is given in Table II.

TABLE II.

DEVELOPMENT OF PROTOZOA IN THE SOIL.

Soil	Moisture	Soil sterilized	Unsterilized, 3 gm. dried blood	Check
Sandy	0.5 optimum	.....	.....	.....
"	1.0 "	.....	.....	.....
"	1.5 "	.....	S.F.	.....
"	2.0 "	F. S.C.	F. S.C.	.....
Loam	0.5 "	.....	.....	.....
"	1.0 "	.....	.....	.....
"	1.5 "	S.F.	S.F.	.....
"	2.0 "	F. S.C.	F.	.....
Clay	0.5 "	.....	.....	.....
"	1.0 "	.....	.....	.....
"	1.5 "	.....	.....	.....
"	2.0 "	F. S.C.	F. C.	.....

F. = Flagellates. S. F. = Small flagellates. C. = Ciliates. S. C. = Small ciliates.

In the table one sees that the protozoa were found to be active with high moisture only where the soils have been sterilized, or when some organic matter has been added to them. No protozoa were found in the untreated soils even with the highest moisture content when the soil was almost saturated. But where organic matter was added, or where the organic matter of the soil was made more available by sterilization under pressure, active protozoa were found at one and one-half times the optimum, in the case of sandy and loam soils, and at two times the optimum in the case of the heavy clay soil. This brings out the fact that the protozoa will become active in different soils under similar treatment at correspondingly different moisture contents. In a lighter soil the pro-

tozoa may develop in the presence of easily soluble plant food at one and one-half times the optimum, which is about 18 to 20 per cent moisture. In a heavy soil at that moisture condition, which may be 30 to 35 per cent, no protozoa will be found. This question of easily available plant food which can be utilized by the protozoa, and hence bring about their greater activity, will be taken up in the third part of this work.

As to the types of protozoa developing in the soil, we conclude that with the methods used, the small flagellates develop as soon as conditions become favorable; these are followed by large flagellates and small ciliates Large ciliates appear only in a few cases where the moisture content of the soil is very high, or other conditions are exceptionally favorable for their development. This confirms the results of some of the other investigators, who have either proven, or else have expressed the opinion, that the small flagellates are probably the active protozoan fauna of the soil, when conditions are nearly normal. Sherman (16) has also expressed the opinion that the active protozoan fauna in most soils is restricted to the flagellates. He found that ciliates become active only in soils containing much more moisture than is required for flagellates. He also shows that when sterilized soil is inoculated with normal soil, the protozoan fauna rises in numbers above that of normal soils, just as does the bacterial flora. Sterilization of the soil and the addition of organic matter seem to act in the same manner as an increase in the moisture content.

The method used seems to be fairly accurate for the detection of flagellates and ciliates in the soil, but it is of very little value for the detection of amoebae. The picric acid method, as outlined by Martin and Lewin (11) is being used for that purpose. Some organisms have been found by this method, which have not been detected in the soil by the ordinary water method; these resemble the thecamoebae described by the previously named writers. Not enough study has yet been made by this method to make it possible to state any definite results.

II.—Numbers and Types of Protozoa in the Soil.

1. *Numbers of Protozoa.*

The dilution method has been used for this part of the work. This was mentioned by Rahn (12) and more fully described by Cunningham (3) for the determination of total numbers of protozoa in the soil. Since all the protozoa do not develop on one medium, and as it is very hard to differentiate in dilution work between cysts and active forms, the total numbers of both cysts and active forms are given. Fifty cubic centimeters of sterile Lohnis' soil extract, in Erlenmeyer flasks, was inoculated with proper dilutions of soil and allowed to stand for five to ten days, then determinations of the presence of protozoa were made. Dilutions made were as follows: 1.0, 0.1, 0.01, 0.001, 0.002, 0.005, 0.0001. Soils used for this

part of the experiment as well as for the second are, 1, 2, 3, soil types used in the previous part of the work, and 4, a permanent forest soil. Samples were taken under sterile conditions, at depths of 1, 4, 8, 12, 20 and 30 inches.

The results are reported in Table III.

TABLE III.

NUMBERS OF PROTOZOA AT DIFFERENT SOIL DEPTHS.

Depth of soil in inches	Soil A				Soil B			
	Protozoa	Moist'e	C%	N%	Protozoa	Moist'e	C%	N%
1	2.000-5.000 F.	9.3	1.56	0.1127	1.000-2.000 F.	9.0	.90	0.1158
4	2.000-5.000 F.	9.4	1.41	0.1176	100-1.000 F.	10.3	.41	0.1071
8	2.000-5.000 F.	9.0	1.76	0.0973	100-1.000 F.	10.0	.44	0.1036
12	10-100 F.	8.0	.57	0.0581	10-100 F.	8.3	.41	0.0819
20	0	9.0	.63	0.0420	0	6.0	.18	0.0413
30	0	10.0	.67	0.0581	0	7.7	.17	0.0287

Depth of soil in inches	Soil C				Soil D			
	Protozoa	Moist'e	C%	N%	Protozoa	Moist'e	C%	N%
1	5.000-10.000 F.	17.3	1.70	0.1918	10-100 F.	23.6	3.38	0.2345
4	1.000-2.000 F.	12.3	1.12	0.1596	10-100 F.	15.3	1.58	0.1015
8	100-1.000 F.	12.3	1.05	0.1267	1-10 F.	10.	0.56	0.0469
12	10-100 F.	11.3	.53	0.1050	0	11.	0.23	0.0294
20	0	12.3	.20	0.0518	0	14.	0.09	0.0294
30	0	13.3	.20	0.0350	0	14.5	0.12	0.0307

F. = Flagellates. C% = Total carbon in per cent. N% = Total nitrogen in per cent.

It is seen from the above table that protozoa are found in the soil between the depts of 1 and 12 inches, the largest numbers occurring just below the surface. The numbers decrease with the depth, and below 12 inches the soil is almost free from protozoa. At times protozoa were found when soil from 20 or even 30 inches below the surface was inoculated into the culture medium, but these cases were exceptional and should not be taken into account. Soil No. 4, which is an acid forest soil, seems to be very poor in numbers. Whether this is due to acidity or other reasons is not known. Ciliates and amoebae are not recorded as they occurred only occasionally. They usually varied from 10 to 100 per gram of soil, and their occurrence as to types will be pointed out later. These results seem to confirm Rahn (12), who found 1,000 to 10,000 flagellates and about 100 ciliates per gram of soil.

2. *Types of Protozoa in the Soil.*

The same soils and the same depths of sampling were used in this experiment. One gram of soil was inoculated into 50 c.c. of sterile soil extract, and records taken after 3, 5, 7, and 12 days incubation. In this part of the work only the types of protozoa developing in the solution will

be given. Details as to the different types of protozoa at different depths of soil and in different soils cannot be given as yet, but from the data collected it is seen that there is a difference among protozoan types in the different soil layers. The ciliates and large flagellates are found in greatest numbers at a depth of 4 inches, while the flagellates are present in largest numbers at a depth of 1 inch. It appears as if the smaller organisms are present in largest possible numbers near the surface, where conditions favor microörganic activities, while the large organisms (e. g. ciliates) are found at a somewhat lower depth, where the moisture conditions may be more favorable for their development. The common types found among the flagellates are *Monas guttula* Ehrbg., *Monas vivipara* Ehrbg., *Bodo ovatus* Stein, *Bodo augustus* Duj., *Chlamydomonas* and *Pleuromonas* types, more seldom the *Euglena viridis, Phyllomitus undulans,* etc. Among the ciliates: *Colpidium colpoda* Ehrbg., *Colpoda cucullis* O. F. M., and *Enchelys pupa* Ehrbg., most common ones, then *Prorodon ovum* Ehrbg., *Nassula elegans* Ehrbg., *Glaucoma* types, *Paramoecium, Pleuronema, Halteria, Uroleptus, Uronema, Strombidium, Chilodon, Oxytricha, Vorticella, Pleurotricha, Euplotes,* and several others.

The data on the occurrence of amoebae are very meagre because the methods used until now could not bring out the actual facts about those organisms. However, work is being done at the present time on that group of organisms.

III.—Effect of Protozoa on Bacterial Numbers and Upon Ammonification in the Soil.

Russell and Hutchinson (11), after a series of experiments with partially sterilized soils, concluded that the bacterial numbers are limited in certain soils by detrimental organisms, which have much in common with protozoa; they also concluded that an increase in the rate of production of ammonia does not take place without bacterial multiplication; and since protozoan activities limit the bacterial numbers in the soil, they also limit the production of ammonia. Cunningham (3) has conducted experiments on "The Influence of Protozoa on the Numbers of Bacteria Developing in Ammonifying Solutions." Into 1 per cent bloodmeal solution plus .05 per cent K_2HPO_4 he inoculated bacteria and protozoa from a culture of protozoa from the soil, and bacteria alone. Counts of the bacteria were made at intervals, and the results show that the soil protozoa, in solutions at all events, exercise a very decided limiting effect on the numbers of bacteria. He determined also the ammonia formed in the solutions with and without protozoa. In one instance no appreciable difference was found in the amount of ammonia formed; he attempts to explain that lack of difference, by the higher original bacterial count of the protozoa cultures. In the other two instances the ammonia accumulated in the pres-

ence of protozoa was smaller than in the solutions where they were absent, although the number of bacteria inoculated into the medium was greater in the case where they were with protozoa. By inoculating protozoan cultures and protozoa-free cultures of bacteria into partially sterilized soil, Cunningham found a great reduction in bacterial numbers where protozoa had been added. From these results he concluded that the presence of protozoa is the limiting factor, or at least one limiting factor, in bacterial numbers and activities in the soil.

The interaction of the bacteria and protozoa in the soil and the ammonia accumulation resulting from the decomposition of the organic matter is not such an easy problem as one might at first regard it. The different kinds of protozoa and bacteria, the type of soil, the treatment of the soil, the amount of moisture present, are all important factors in determining the ammonia accumulated in a given soil, which has been inoculated with protozoa and bacteria. One of the most obvious questions is, How does moisture influence the interaction between protozoa and bacteria? As it was shown in the early part of the work, moisture plays a very important part in the activity of protozoa; below a certain moisture content the protozoa will not be active (at least the author has not been able to find it so). The more moisture added to the soil, the more favorable the conditions become for the growth of protozoa. Protozoa develop best where the soil is fully saturated with water. If protozoa limit bacterial numbers and their activities in the soil, resulting in a decrease in ammonia accumulation, the amount of moisture added to a certain soil inoculated with a certain number of protozoa and bacteria, ought to influence the bacterial numbers and the amount of ammonia accumulated in the soil. By starting with a soil of low moisture content and adding moisture to it, the conditions should be made less favorable for bacterial development and ammonia accumulation where the protozoa are present, than where they are absent.

METHODS—100 gm. of air-dried soil (Sassafras loam was used throughout, with a physical optimum moisture of 15 per cent) and 155 mg. of nitrogen in the form of dried blood were placed in 250 c.c. Erlenmeyer flasks, and the proper amount of water added. The flasks were plugged and sterilized at 15 pounds for 15 minutes. One-c.c. portions from bacterial cultures free from protozoa and bacteria plus protozoa cultures were added to the flasks, which were then incubated for 7 days at 22° C. The culture of protozoa plus bacteria was made by inoculating some soil into Löhnis' soil extract. Protozoa-free cultures of bacteria were made by inoculating soil taken 20 inches deep and free from protozoa. Cultures were kept from 8 to 10 days and before being used for inoculation they were carefully examined as to the presence or absence of protozoa. The protozoa were counted with the microscope by the direct counting method,

and were found to be 100,000 to 200,000 per c.c. The numbers of bacteria were determined by plating out on Brown's albumen agar. The ammonia was determined by distilling the soil with MgO. The results are shown in Table IV.

TABLE IV.

Mg. OF NITROGEN ACCUMULATED AS AMMONIA.*

Inoculum taken from	Moisture in per cent	Bacteria alone			Protozoa + Bacteria		
		Bacteria per c.c. Inoculum	Mg. N as Ammonia	Average	Bacteria per c.c. Inoculum	Mg. N as Ammonia	Average
Soil A	16.	17,200,000	34.01 29.31	31.66	35,000,000	27.12 20.90	24.02
"	32.	"	36.22 38.13	37.18	"	58.04 50.12	54.08
Soil B	16.	32,500,000	16.38 13.58	14.98	13,000,000	17.70 15.64	16.67
"	32.	"	14.76 15.93	15.35	"	30.28 24.38	27.33

One can see from the above table that the increase in moisture gives an increase in ammonia production in both cases, but where the protozoa are present the results are somewhat striking. One might have expected that with an increase in moisture the activity of the protozoa would check the bacterial activities, but the results do not verify that. With soil A twice as many bacteria have been added to the soil in the protozoa culture as in the protozoa-free culture, and though at 16 per cent moisture the ammonia produced is less where the protozoa were present than where they were absent, at 32 per cent moisture the ammonia accumulated in the presence of protozoa is more than twice as great as with 16 per cent moisture, while the bacteria alone gave only a slight increase with the increase in the moisture content of the soil. The same holds true with the soil portions inoculated with the organisms isolated from soil B (unfertilized Sassafras loam), but in this case fewer bacteria had been added to the soil in the protozoa cultures. The soil portions, before the ammonia has been distilled off, were examined for living protozoa; small flagellates were found in an active state at the lower moisture content of the soil, and both ciliates and flagellates were found at the higher moisture content. It appears that the activities of protozoa stimulated the decomposition of organic matter in the soil, when the conditions were favorable for their development.

* In this experiment as well as in all the subsequent experiments a check was used, consisting of the same amount of soil with the same quantity of organic matter, but not inoculated. This has been kept under the same conditions of moisture and temperature with the inoculated soil portions. The check, giving 2 to 3 mg. N, was always subtracted from the determinations, in order to eliminate the ammonia accumulated due to the decomposition of the organic matter by sterilizing the soil.

To be able to study more closely the effect of moisture upon the activities of the microörganisms in the soil, another experiment was started, in which different degrees of moisture were used. The same soil and organic matter and the same incubation period were used in the following experiment as in the previous one.

TABLE V.

Mg. OF NITROGEN AS AMMONIA IN THE SOIL.

Inoculum taken from	Moisture in per cent	Bacteria alone			Protozoa + Bacteria		
		Bacteria per c.c. Inoculum	Mg. N as Ammonia	Average	Bacteria per c.c. Inoculum	Mg. N as Ammonia	Average
Soil A	7.	4,000,000	3.09 2.79	2.94	7,200,000	3.09 3.68	3.38
"	14.	"	6.91 6.91	6.91	"	9.11 10.88	10.00
"	28.	"	38.22 30.42	34.32	"	30.22 25.64	27.93
"	42.	"	3.23 4.41	3.82	"	3.97 4.70	4.34
Soil B	7.	18,000,000	2.06 2.35	2.21	28,000,000	2.06 1.76	1.91
"	14.	"	10.44 9.12	9.78	"	20.93 16.80	18.87
"	28.	"	16.02 13.82	14.92	"	24.55 23.23	23.89
"	42.	"	2.35 2.57	2.46	"	14.60 17.64	16.12

Table V shows that the presence of protozoa not only did not decrease the ammonification by bacteria when the moisture conditions became favorable for their development, but in some instances favored it. In looking through the columns one can see a parallel increase in ammonia accumulation as the moisture was increased from 7 per cent to 28 per cent, then the amount of ammonia accumulated drops down in all instances because at 42 per cent there was a free layer of water on the surface of the soil. The soils were examined for their protozoa content. No living protozoa were found in the soil that contained the 7 per cent moisture, small flagellates at 14 per cent, flagellates and ciliates at 28 per cent, flagellates and small ciliates at 42 per cent. With the increase in moisture content the protozoa became more active, but they did not seem to influence the amount of ammonia accumulated, and if they did have any influence, it was only beneficial.

One c.c. of inoculum was used in the experiments previously cited.

To see whether the numbers of bacteria and protozoa added might have any influence upon the ammonia accumulated, it was thought advisable to double the bacterial or protozoan numbers. Bacterial numbers in the soils were determined at the end of the seven-day incubation period by plating out the desired dilution on Brown's albumen agar. (2)

TABLE VI.

Mg. OF NITROGEN AS AMMONIA AND NUMBERS OF BACTERIA IN THE SOIL.

Moisture in per cent	Inoculum	Bacteria alone 12,000,000 bacteria per c.c. Inoculum				Protozoa + Bacteria 13,000,000 bacteria per c.c. Inoculum			
		N as Ammonia		Bacteria per gm. of soil		N as Ammonia		Bacteria per gm. of soil	
		Mg. N	Average	Nos. found	Average	Mg. N	Average	Nos. found	Average
7	1 c.c.	2.65 2.79	2.72	34,000,000 38,000,000	36,000,000	3.53 3.67	3.60	28,000,000 24,000,000	26,000,000
7	2 c.c	2.94 2.82	2.88	.....	.....	3.38 3.24	3.31	.....	.....
14	1 c.c.	11.91 11.31	11.61	500,000,000 526,000,000	513,000,000	15.32 18.93	17.13	472,000,000 512,000,000	494,000,000
14	2 c.c.	14.70 15.28	14.99	.....	.....	23.67 19.99	21.83	.....	.....
28	1 c.c.	40.28 39.98	40.13	450,000,000 470,000,000	460,000,000	30.30 37.04	33.67	230,000,000 156,000,000	193,000,000
28	2 c.c.	37.19 40.72	38.96	.....	.....	33.22 31.32	32.27	.....	.....
42	1 c.c.	5.00 5.00	5.00	50,000,000 44,000,000	47,000,000	14.99 15.29	15.14	110,000,000 112,000,000	111,000,000
42	2 c.c.	5.73 4.56	5.15	.....	.....	15.29 14.85	15.07	.....	.....

The addition of double portions of the inoculum did not influence the results appreciably. In a few cases there is a slight increase in the ammonia accumulated, while in other cases there is a slight decrease. The presence of protozoa did not influence the ammonification in the soil with the increase in moisture. The records of bacterial numbers show an increase at from 7 per cent to 14 per cent of moisture, a slight decrease at 28 per cent, and a drop at 42 per cent. The drop between 14 per cent and 28 per cent is much greater in the cultures where protozoa were present. It is interesting to note that in both cases the amounts of ammonia produced do not correspond to the bacterial numbers; the largest bacterial numbers are found in both cases at 14 per cent of moisture, while the highest ammonia accumulation is found in both cases at 28 per cent.

In all the previous experiments the bacteria used for protozoa-free

culture were taken from the subsoil, and though the results are to be compared only within the same inoculum under varying moisture conditions, some objections might be raised to the use of different types of bacteria from those used in protozoa cultures. To eliminate this objection, fresh cultures were prepared by inoculating into 100 c.c. of soil extract 1 gm. and 0.00001 gm. of soil respectively. The cultures were examined after several days. Those that were inoculated with 1 gm. of soil contained bacteria and protozoa; those that were inoculated with 0.00001 gm. contained only bacteria. These cultures were now used for the work. Objections have also been raised by Russell (14) against the seven-day period of ammonification used by Lipman and his associates; he claimed that the results of the activities of the protozoa (e. g. detrimental factor) cannot be seen in such a short period of time. The following experiments were carried out for 14 and 30 days, while bacterial counts were taken 5, 7, 14 and 30 days after incubation. Though only .00001 gm. of soil was used for inoculation for the protozoa-free culture, the culture contained after 6 days 161,000,000 bacteria per c.c. as compared with 6,500,000 bacteria per c.c. where 1 gm. of soil was used for incubation. This great reduction in bacterial numbers might be due to the presence of protozoa in the second culture. The same amounts of soil and organic matter as in the previous experiments and 1 c.c. inoculum have been used in the following experiment.

TABLE VII.

Mg. OF NITROGEN AS AMMONIA IN THE SOIL.

Moisture in per cent	Incubation	Bacteria 161,000,000 per c.c.		Protozoa + Bacteria 6,500,000 bacteria per c.c.	
		Mg. N	Average	Mg. N	Average
7	14 days	5.09 8.32	6.71	7.44 6.56	7.00
14	14 days	71.61 78.15	76.38	73.29 82.55	77.92
28	14 days	76.38 77.70	77.04	87.85 86.08	86.97
42	14 days	10.38 10.02	10.20	9.35 11.70	10.53
7	30 days	14.35 20.37	17.36	29.64 24.48	27.06
14	30 days	72.41 70.21	71.31	67.56 69.32	68.44
28	30 days	76.82 79.44	78.13	76.09 75.06	75.58
42	30 days	7.00 5.52	6.26	15.67 13.02	14.35

Table VII gives the ammonia that had accumulated in the cultures after 14 and 30 days respectively. The facts brought out in the previous experiments hold true also in this one; the increase in moisture gave a higher ammonia accumulation up to double the optimum, the moisture that resulted in a decrease. The presence of protozoa did not affect in any way the ammonia accumulated in the cultures. But turning to Table VIII, where the bacterial numbers of the same cultures are given for different periods, one finds a great difference between the cultures containing protozoa and those free from those organisms. The protozoa-free cultures contained after a five-day period of incubation 18 to 325 millions per gram, the numbers varying with the moisture content of the soil, the highest numbers being found with 28 per cent of moisture, and the lowest with 7 per cent. After 7 days inoculation, the bacterial numbers greatly increased, the highest numbers, 1536 millions per gram of soil, occurring with 14 per cent moisture and the lowest with 42 per cent. After the seven-day period the bacterial numbers decreased steadily, but the relation between the different moistures holds true also with the fourteen- and thirty-day periods: the highest bacterial numbers occurring with 14 per cent or 28 per cent, the lowest with 7 per cent or 42 per cent.

TABLE VIII.

BACTERIAL NUMBERS IN THE SOIL, IN MILLIONS PER GRAM.

Moisture in per cent	Bacteria alone used as Inoculum								Protozoa + Bacteria							
	5 days		7 days		14 days		30 days		5 days		7 days		14 days		30 days	
	Bacteria	Average	Bacteria	Average	Bacteria	Average	Bacteria	Average	Bacteria	Average	Bacteria	Average	Bacteria	Average	Bacteria	Average
7	14. 22.	18.	596. 582.	589.	612. 604.	608.	30. 33.	31.5	176. 186.	181.	204. 226.	215.	260. 224.	242.	440. 530.	485.
14	26. 36.	31.	1580. 1492.	1536.	828. 638.	730.	100. 90.	95.	148. 144.	146.	298. 312.	305.	138. 152.	145.	74. 46.	60.
28	314. 336.	325.	972. 878.	925.	885. 864.	875.	60. 72.	66.	102. 86.	94.	142. 128.	135.	162. 124.	142.	54. 60.	57.
42	216. 206.	211.	320. 296.	308.	660. 685.	673.	28. 28.	28.	42. 48.	45.	36. 28.	32.	108. 102.	105.	42. 52.	47.

When the bacterial numbers of the protozoa plus bacteria cultures are compared with those of the protozoa-free culture, a great difference is found; while the latter contained the highest bacterial numbers at the optimum moisture conditions which seemed to vary between 14 per cent and 28 per cent, and the lowest numbers with the 7 per cent and 42 per cent of moisture; where the protozoa were present the bacterial numbers were highest with 7 per cent moisture, and only in one case with 14 per

cent; the numbers of bacteria then rapidly decreasing with the increase in moisture. The 7 per cent moisture content should have given the lowest bacterial numbers as one could have expected from the results of the ammonia accumulation and the bacterial numbers in the protozoa-free cultures. It seems as if the increase in moisture, making the conditions more favorable for the protozoa development, had, at the same time, a detrimental effect upon the bacterial numbers. Upon examining the soils with a microscope, the protozoa were not found in an active state with the 7 per cent moisture, but were found leading a trophic life in cultures containing 14 per cent moisture and more.

This experiment seems to bear out the conclusions of Russell and Hutchinson that the protozoa are one of the factors detrimental to bacterial numbers. But it does not bear out their second conclusion, that the increase in ammonia production was due to the destruction of the detrimental organisms and the rise in bacterial numbers. Although the bacterial numbers were greatly decreased in the presence of protozoa when the conditions became favorable for their development, the ammonification was not affected at all. It appears that either the bacteria destroyed played no part at all in the ammonia accumulation, or that the protozoa, or some types of protozoa, took part in the decomposition of the organic matter and the accumulation of ammonia. A suggestion with respect to the possible ammonification by protozoa was made in 1896 by Bréal (14).

To see whether the presence of protozoa can have any effect at all upon bacteria having a strong ammonifying power, the following experiment was started. A series of flasks containing 100 gm. of soil, 155 mg. of nitrogen as dried blood, and different quantities of moisture, were sterilized, then inoculated with 1 c.c. of a three-day-old culture of *B. mycoides* and protozoa plus bacteria culture grown for 10 days in soil extract. The flasks were incubated at 22° to 25° C, and duplicate ammonia determinations made after 6, 20 and 40 days.

The presence of protozoa not only did not decrease the ammonia accumulated by the *B. mycoides,* but in some instances a strong associative action between the different organisms is found, and the protozoa, at least as far as ammonification is concerned, do not have any detrimental effect at all.

Russell (14) in his answer to his American critics states again, "The increased ammonia production is attributed to the increased numbers of bacteria. The factor limiting bacterial numbers in ordinary soils is not bacterial, nor is it any product of bacterial activity, nor does it arise spontaneously in soils. The effect is rather variable, but is usually most marked in moist soils that have been well supplied with organic manures. The detrimental organism develops more slowly than bacteria . . . . and causes a marked reduction in the numbers of bacteria."

TABLE IX.

Mg. OF NITROGEN AS AMMONIA IN THE SOIL.

Moisture in per cent	Incubation	B. mycoides		Protozoa + Bacteria		B. mycoides + Protozoa	
		Mg. N	Average	Mg. N	Average	Mg. N	Average
10	6 days	3.48 3.32	3.40	0.82 1.12	0.97	2.29 2.73	2.51
10	20 days	6.20 5.67	5.94	0.38 0.02	0.20	8.91 9.02	8.97
10	40 days	6.26 6.85	6.56	5.82 4.06	4.94	10.97 13.46	12.22
25	6 days	4.21 4.94	4.58	2.00 2.59	2.30	5.53 6.82	6.18
25	20 days	14.93 15.24	15.09	15.61 16.03	15.82	60.79 58.89	59.84
25	40 days	76.67 80.49	78.58	89.90 88.82	89.36	94.02 93.46	93.74

These conclusions have to be taken with a great deal of care in the light of the present experiments. The results brought out in this paper show that though the protozoa may be detrimental to bacterial numbers, they do not influence the ammonia accumulated in the soil, a fact which is the important part of the question, and with which we concern ourselves in studying the problems of soil fertility. The question whether the protozoa do play any part in the fertility of the soil can be answered only after sufficient work has been done with protozoa in their interaction with the soil bacteria.

The presence of protozoa seems to be detrimental to bacterial numbers. But, either the bacteria destroyed do not take any active part in the ammonification, or the protozoa, destroying some bacteria, influence beneficially the decomposition of organic matter. Culture solutions containing protozoa have a more pleasant odor than those containing bacteria alone; it appears as if the protozoa either destroy the decomposition products or the putrefactive organisms. These facts lead to the following question, "Are not the protozoa natural and necessary factors in the fertility of the soil?"

Since the completion of this work for publication, the issue of the Experiment Station Record, Vol. XXXIII, No. 6, has come to hand. This contains abstracts of articles dealing in one way or another with soil protozoa. This work has been done by two of the first and foremost students on protozoa and their relation to bacterial activities. These are Goodey and Hutchinson. In his "Investigations on Protozoa in Relation to the Factor Limiting Bacterial Activity in the Soil," Goodey (7) comes to the follow-

ing conclusions: "The protozoa, including ciliates, amoebae and flagellates added to the soil have not been able to act as a factor limiting bacterial activity in the soil. Inferentially, therefore, the ciliates, amoebae, and flagellates obtainable from ordinary soil under cultural conditions do not function as the limiting factor." Hutchinson (8) in his work on green manures says: "The rapid ammonification which takes place when green manure is placed in water and allowed to ferment was found to be accompanied by the development of large numbers of ciliates, flagellates, and amoebae, whose presence does not appear in this instance to be prejudicial to the activity of ammonifying bacteria."

But if the protozoa play a doubtful part in the fertility of normal soils, might not they play an important rôle in specialized soils, which may become "sick" or "tired" after being subjected to the same treatment for a number of years? In those soils certain types of protozoa might develop which would become detrimental factors in the fertility of those soils. At the suggestion of Dr. Löhnis, the writer has undertaken to create the "sick" or "tired" condition of the soil artificially in the laboratory by treating soils with high quantities of manure, keeping them under wide ranges of moisture and temperature, and studying the types and activities of the protozoa that develop under those conditions.

The work last outlined, together with the isolation of different types of protozoa free from bacteria, and the working out of different methods for the study of protozoa in the soil, form the main lines of present investigations by the writer, in the field of soil protozoology.

SUMMARY.

1. Moisture, humus content and the structure of the soil are the important factors governing the activities of the protozoa.

2. Sterilization of soil and addition of easily soluble organic matter will make the conditions optimum for protozoan activities at lower moisture content than the corresponding unsterilized or untreated soils.

3. The flagellates are the most common soil protozoa, found active in the soil with moisture content too low for the development of the other groups.

4. The flagellates are the largest group of soil protozoa; the greatest number of flagellates are found in the soil just below the surface; the ciliates at a depth of 4 inches; the numbers decrease with the depth, so that below 12 inches the soil is practically free from protozoa.

5. Soil protozoa do not have any appreciable influence upon the ammonification by bacteria.

6. The presence of protozoa acts detrimentally upon bacterial numbers, so that when the conditions become favorable for protozoa development, the bacterial numbers decrease.

7. The detrimental effect of the protozoa upon the bacterial numbers and their non-detrimental, and even beneficial effect at times upon the soil, and their influence upon ammonification in the soil, might be explained by one of the following assumptions: (1) if the protozoa destroy bacteria, they destroy non-ammonifying organisms; (2) the protozoa themselves take part in the process of ammonification; or (3) the disintegration of the bacterial cells results in decomposition products which might be responsible for high ammonia production.

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QUANTITATIVE MEDIA FOR THE ESTIMATION OF BACTERIA IN SOILS.*

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A study of bacteriology in the estimation of the number of bacteria in soils seems to be of considerable value when taken together with other factors. Not only the number, but also the character of the organisms present, must exert greater or less influence upon soil fertility, and a study of both is essential to one's understanding of the problems involved.

Of the methods which have been devised for use in counting soil bacteria, the plate method is without doubt the most satisfactory. But the choice of a good medium is of prime importance. Among the numerous formulae suggested up to the present time there are few which are not seriously lacking in one or more respects. Quantitatively, the medium which will permit the development of the maximum number of colonies is usually most satisfactory. In order, too, that the results be comparable at different times and different places, it is desirable that the constituents be of definite chemical composition, or that the medium be made up synthetically. For qualitative work, however, definiteness of composition assumes less prominence and admits of greater choice in the selection of materials.

Recent contributions to the subject by Brown (1) and Conn (2) have indicated progress in both quantitative and qualitative directions. Earlier work by Temple (5), by Lipman and Brown (4), and by Fischer (3), forms a basis of comparison.

It was the purpose of these experiments, besides corroborating some of the work noted above, to contribute something toward the improvement of present facilities.

With this in mind, experimental work was outlined in which particular attention was directed to a comparison of a number of media which have been in use for some time in bacteriological studies. A few variations and additions were planned and carried out as noted below.

The comparisons of the various media were made from at least two different preparations upon several soils. One preparation of media or one or two soils do not seem to be a sufficient test upon which to base a correct interpretation of results. It is evident from a study of the tables following that not all soils behave toward the various media to a like extent, or even, at times, in the same direction in the comparative tests.

* Received for publication August 1, 1915.

The agar used throughout the experiments was thoroughly mixed so that the possibility of variation at this point was slight. In every case the nitrogenous material was added just before sterilization. All plates were poured in triplicate with dilutions of 60,000, and subsequently incubated at a temperature of about 22° C.

Six types of fresh soil, designated respectively by the Bureau of Soils as Penn Loam, Penn Sandy Loam, Sassafras Gravelly Loam, Sassafras Sandy Loam, Sassafras Silt Loam, and Alloway Clay, were used. In order to test the media upon widely varied soil conditions, samples were secured, so far as possible, from plots receiving different treatments.

TABLE I.

COMPOSITION OF MEDIA USED IN THE COMPARATIVE TESTS.

MEDIA Constituents	Liters Distilled Water	Liters Tap Water	Gm. Agar.	Gm. K_2HPO_4	Gm. $NH_4H_2PO_4$	Gm. $MgSO_4$	Gm. Dextrose	Gm. Peptone	Gm. Albumen	Gm. Casein	Gm. Urea	Gm. Asparagine	Gm. $CaCl_2$	Gm. KCl	Gm. NH_4NO_3	Gm. FeCl$_3$	Gm. $Fe_2(SO_4)_3$	Gm. Sod. Asparaginate	c.c. Hay Infusion	c.c. Bloodmeal Extract
I. Lipman and Brown's Modified Synthetic Agar	1.0		15	.5		.2	10	.05									Tr.			
II. Conn's Sodium Asparaginate Agar*	1.0		12		1.5	.2	1						.1	.1		Tr.		.5		
III. Brown's Urea Agar	1.0		15	.5		.2	10				.05						Tr.			
IV. Brown's Asparagine Agar	1.0		15	.5		.2	10					.05					Tr.			
V. Brown's Casein Agar	1.0		15	.5		.2	10			.1							Tr.			
VI. Brown's Albumen Agar	1.0		15	.5		.2	10		.1								Tr.			
VII. Albumen Agar	1.0		15	.5		.2	10		.2								Tr.			
VIII. Albumen Agar	1.0		15	.5		.2	10		.3								Tr.			
IX. Albumen Agar	1.0		15	.5		.2	10		.4								Tr.			
X. Bloodmeal Agar	.92		15	.5		.2	10										Tr.			80§
XI. Hay Infusion Agar	.94		15	.5		.2	10										Tr.		60†	
XII. Temple's Peptone Agar		1.0	15					1												
XIII. Albumen Agar	1.0		15	.5		.2	10		.1*								Tr.			
XIV. Albumen Agar	1 0		15	.5		.2	10		.2*								Tr.			
XV. Albumen Agar	1.0		15	.5		.2	10		.3*								Tr.			
XVI. Albumen Agar	1.0		15	.5		.2	10		.4*								Tr.			
XVII. Urea-Ammonium Nitrate Agar	1.0		15	.5		.2	10				.05				.05		Tr.			
XVIII. Urea-Ammonium Nitrate Agar	1.0		15	.5		.2	10				.05				.1		Tr.			
XIX. Urea-Ammonium Nitrate Agar	1 0		15	.5		.2	10				.05				.5		Tr.			
XX. Urea-Ammonium Nitrate Agar‡	1.0		15	.5		.2	10				.05				.1		Tr.			

* Dissolved in 1 c.c. $\frac{n}{5}$ NaOH and 5 c.c. water.

* Brought to .8% acid.

‡ Brought to .25% acid.

§ Prepared by digesting 10 gm. bloodmeal at 100° C. in 100 c.c. of water for one hour and filtering.

† Prepared by digesting 10 gm. of timothy hay in 100 c.c. of water at 100° C. for one hour and filtering.

Since the nature of the nitrogenous material seems to exert considerable influence upon the development of colonies (1), it was thought that possibly the substitution of certain new substances might be advantageous. A few preliminary trials with an extract of bloodmeal and hay infusion as a source of nitrogen had given promising results, but the variability in composition and difficulty of preparation made them unsatisfactory for use in quantitative media. For qualitative purposes they show up well and the differentiation in colony growth is pronounced. They were included in the first two tests.

The albumen agar suggested by Brown was subjected to some changes. For .1 gm. of egg albumen, as is recommended, .2 gm., .3 gm. and .4 gm. were substituted in an effort to determine if there might not be a point between .1 gm. and .5 gm. where a larger number of colonies might develop.

The composition of all media used in the experiments is given in the following table; except where otherwise noted, the reaction was not adjusted.

Series No. 1.

In the first series twelve media were compared on four soils, Lipman and Brown's modified synthetic agar being taken as a basis of comparison. Sterilization was accomplished in flowing steam for one hour and fifteen minutes.

A comparison of Tables II and III shows that there is a decided increase between the third and fifth day. While varying in extent with different media; this increase averages about 75 per cent. It should be observed also that the relative bacterial numbers as indicated by the counts on the third day is not quite the same as shown by the longer incubation period.

With one exception, Table III shows the sodium asparaginate agar to have given the highest counts. Brown's albumen agar, Temple's peptone agar and the bloodmeal agar having shown up well. With Soil No. 3 Brown's albumen agar stood first by a small margin. The differences between the various media are, however, less pronounced than might be expected. In fact it would be difficult to differentiate between some of them from the counts secured.

The rather inconsistent counts secured with the albumen agar may be due to lack of thorough distribution of the coagulated material even though the tubes were shaken before pouring the plates.

TABLE II.—THREE-DAY INCUBATION PERIOD.†

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.*

Media	Soil No. 1 Penn Loam	Soil No. 2 Penn Sandy Loam	Soil No. 3 Sassafras Sandy Loam	Soil No. 3 Alloway Clay
I. Lipman & Brown's Modified Synthetic Agar..	5.49	5.01	3.46	3.01
II. Conn's Sodium Asparaginate Agar	7.65	6.48	3.56	4.50
III. Brown's Urea Agar.....	6.12	4.09	3.43	3.84
IV. Brown's Asparagine Agar...	4.42	3.63	2.47	2.80
V. Brown's Casein Agar.....	6.64	6.11	3.44	4.68
VI. Brown's Albumen Agar....	6.77	5.53	3.47	4.87
VII. Albumen Agar.....	6.93	5.67	3.30	4.80
VIII. Albumen Agar.....	6.40	5.14	3.27	4.88
IX. Albumen Agar.....	6.77	5.86	3.35	4.83
X. Bloodmeal Agar.....	7.05	5.84	3.72	5.27
XI. Hay Infusion Agar.....	5.12	4.89	2.58	3.37
XII. Temple's Peptone Agar.....	5.95	4.85	3.42	3.27

† For individual counts in all cases see corresponding tables at the end.

* Calculated on assumed moisture content of fresh soil.

TABLE III.—FIVE-DAY INCUBATION PERIOD.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media	Soil No. 1	Soil No. 2	Soil No. 3	Soil No. 4
I. Lipman & Brown's Modified Synthetic Agar.	9.28	7.80	7.22	6.32
II. Conn's Sodium Asparaginate Agar.....	11.98	9.58	7.09	8.23
III. Brown's Urea Agar.....	9.77	8.41	6.03	6.72
IV. Brown's Asparagine Agar...	8.64	6.86	4.71	5.07
V. Brown's Casein Agar.....	10.17	9.26	7.20	8.10
VI. Brown's Albumen Agar....	10.63	9.24	7.51	8.05
VII. Albumen Agar.....	10.15	9.12	7.22	7.96
VIII. Albumen Agar.....	10.37	9.11	7.04	8.09
IX. Albumen Agar.....	10.03	9.10	6.73	7.59
X. Bloodmeal Agar.....	11.64	9.24	6.99	8.14
XI. Hay Infusion Agar.....	9.69	7.78	6.26	5.94
XII. Temple's Peptone Agar.....	10.82	8.47	7.25	6.74

Series No. 2.

Series No. 2 was essentially the same as Series No. 1. However, sterilization of all media except the casein and albumen agars was accomplished under steam pressure of one atmosphere for 15 minutes, the casein and albumen agars having been sterilized as before. In an attempt to eliminate the difficulty due to the coagulation of the albumen, a solution of it in 1 c.c. $\frac{N}{6}$ NaOH and 5 c.c. of water was made for media numbers XIII, XIV, XV and XVI. The addition of this solution caused no perceptible coagulation even upon sterilization, and it was found possible to add the material to the medium without cooling. This procedure greatly simplified the preparation of the albumen agar.

TABLE IV.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media	Soil No. 5 Penn Sandy Loam	Soil No. 6 Alloway Clay	Soil No. 7 Sassafras Silt Loam	Soil No. 8 Penn Loam
I. Lipman & Brown's Modified Synthetic Agar.	6.29	6.93	3.62	11.83
II. Conn's Sodium Asparaginate Agar.....	5.95	8.87	4.59	12.23
III. Brown's Urea Agar.....	4.66	7.94	4.04	10.78
IV. Brown's Asparagine Agar...	3.98	6.02	3.63	9.89
V. Brown's Casein Agar.....	5.73	6.63	3.53	11.40
VI. Brown's Albumen Agar....	6.49	7.38	4.50	11.78
X. Bloodmeal Agar.....	6.94	7.92	3.71	11.50
XI. Hay Infusion Agar.....	4.83	7.61	3.02	9.46
XII. Temple's Peptone Agar.....	6.63	7.29	3.70	11.95
XIII. Albumen Agar.....	6.98	8.82	4.37	12.10
XIV. Albumen Agar.....	7.04	8.57	4.21	11.63
XV. Albumen Agar.....	6.92	7.31	4.22	11.14
XVI. Albumen Agar.....	6.59	7.48	4.21	11.77

It is evident that the relationships shown in Series No. 1 are apparently the same in this, the different forms of sterilization having exerted no appreciable influence. The albumen agars do not seem to have deteriorated by the solution of the albumen in sodium hydroxide, but the counts are much less variable and slightly higher than with No. VI. No consistent differences appeared either here or in Series No. 1 between the media having respectively .1 gm., .2 gm., .3 gm., and .4 gm., of albumen.

Series No. 3.

Numbers III, IV, V, VII, VIII, IX, X, XI, XIV, XV and XVI did not warrant further investigation, and in Series No. 3 a comparison of five media was made. With the exception of Brown's albumen agar VI, all were sterilized at 15 pounds for fifteen minutes.

TABLE V.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media	Soil No. 9 Penn Loam	Soil No. 10 Penn Sandy Loam	Soil No. 11 Sassafras Silt Loam	Soil No. 12 Alloway Clay
I. Lipman & Brown's Modified Synthetic Agar.	6.37	4.34	4.91	7.63
II. Conn's Sodium Asparaginate Agar.....	7.49	4.20	6.23	9.75
VI. Brown's Albumen Agar....	7.11	4.27	5.86	9.29
XII. Temple's Peptone Agar.....	6.59	4.11	5.11	8.28
XIII. Albumen Agar.....	7.39	4.52	6.13	9.67

Here again the asparaginate agar gave the highest counts in three out of four cases, albumen agar XIII giving the highest count in one case. Moulds did not develop as readily upon the asparaginate agar as upon

most of the others tried and counting was rarely rendered difficult. However, there are such small differences between the asparaginate and albumen agars that the choice of one over the other would be determined by other factors than mere colony development. But apparently the use of sodium asparaginate will greatly limit the number of disturbing elements in plate counting.

At this point it seemed worth while to determine the effect of the introduction of several forms of nitrogen into a medium. It seems reasonable to expect that with the use of nitrogen from several sources, a larger number of bacteria might develop colonies. The use of NH_4NO_3 suggested itself as furnishing two forms, and a third was taken in urea which had been observed in Tables III and IV to give comparatively high counts. Accordingly, the media XVII, XVIII, and XIX were made up and compared with asparaginate and albumen agars.

TABLE VI.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media	Soil No. 13 Penn Loam	Soil No. 14 Alloway Clay	Soil No. 15 Sassafras Gravelly L'm	Soil No. 16 Sassafras Silt Loam
II. Sodium Asparaginate Agar.	15.97	7.35	3.55	9.88
XIII. Albumen Agar.....	15.50	7.73	2.68	8.95
XVII. Urea-Ammonium Nit. Agar	14.20	8.44	2.99	7.47
XVIII. Urea-Ammonium Nit. Agar	17.30	8.80	3.92	10.41
XIX. Urea-Ammonium Nit. Agar	15.23	7.40	2.55	8.95

Table VI shows that with the four soils used the urea-ammonium nitrate agar containing .05 gm. of urea and .1 gm. of ammonium nitrate gave higher counts than any of the other media. There was, however, a tendency toward mould development, and in an effort to discourage this, Series No. 5 was started in which the reaction of the urea-ammonium agar was changed to .2½ per cent acid in Medium No. XX.

TABLE VII.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media	Soil No. 17 Penn Loam	Soil No. 18 Penn Sandy Loam	Soil No. 19 Sassafras Silt Loam	Soil No. 20 Alloway Clay
II. Sodium Asparaginate Agar.	13.57	6.48	2.21	5.03
XVIII. Urea-Ammonium Nit. Agar	16.02	7.10	2.42	4.89
XX. Urea-Ammonium Nit. Agar	14.95	7.04	3.15	4.98

Series No. 5.

The change in the reaction did not seem to impair the efficiency of the urea-ammonium nitrate agar but rendered it decidedly less favorable to moulds. The relationship to the sodium asparaginate agar was again checked up, tending to show that the urea-ammonium nitrate agar will

permit the development of as many colonies as, or more than, the former; it is also easier to prepare and is much less expensive.

Tests of other combinations would no doubt result in the accumulation of valuable information, but it seems that the media question may be most satisfactorily solved by the use of differential media which will allow the development of particular species of organisms rather than by an attempt to determine the greatest number regardless of species or groups. This should give, in addition to relative bacterial numbers, certain indications concerning the biological activities in different soils.

SUMMARY.

The results of the present work indicate that:

1. Sodium asparaginate agar, albumen agar, and urea-ammonium nitrate agar will in most cases give a greater colony development than other media in common use for bacteriological work.

2. The albumen agar, in which the albumen is dissolved in NaOH, will give more consistent results than if the albumen is used in water solution.

3. A five-day incubation period gave considerably higher bacterial counts than a three-day incubation period.

4. Sterilization either by flowing steam or at a pressure of one atmosphere will give equally good results.

5. Differentiation in bloodmeal and hay infusion agars is marked.

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In the following tables the separate determinations are given, from which the averages set forth in the foregoing tables were derived.

TABLE II-A.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media.	Soil No.-1			Soil No. 2			Soil No. 3			Soil No. 4		
I.	5.14	5.23	6.10	5.30	5.01	4.73	3.21	3.71	3.46	3.07	3.22	2.74
II.	7.32	8.01	7.61	6.82	6.62	5.99	3.76	3.39	3.52	5.66	4.18	3.66
III.	6.16	6.40	5.80	3.59	4.18	4.49	3.14	3.68	3.47	4.03	3.72	3.77
IV.	4.19	4.82	4.26	3.81	3.62	3.45	3.06	2.14	2.22	2.79	3.06	2.55
V.	6.62	6.59	6.91	6.77	5.18	6.37	3.55	3.16	3.62	4.69	5.27	4.09
VI.	6.99	6.03	7.29	5.61	4.88	6.11	3.01	3.82	3.59	4.96	5.53	4.11
VII.	7.30	7.00	6.50	5.19	6.27	5.55	2.98	3.46	3.45	5.18	4.17	5.06
VIII.	6.08	6.82	6.31	4.39	5.72	5.31	3.61	2.89	3.32	4.26	5.29	5.10
IX.	7.11	6.81	6.40	5.66	5.92	6.01	3.53	3.21	3.30	3.99	5.33	5.18
X.	7.24	6.82	7.08	6.77	5.14	5.62	3.49	3.76	3.92	5.17	5.38	5.26
XI.	5.02	5.45	4.90	4.97	4.38	5.33	2.95	2.20	2.60	2.98	3.30	3.82
XII	6.40	6.04	5.42	5.08	4.39	5.08	3.41	3.02	3.82	3.06	3.66	3.10

TABLE III-A.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media.	Soil No. 1			Soil No. 2			Soil No. 3			Soil No. 4		
I.	9.91	8.63	9.30	7.82	7.58	7.99	7.01	7.50	7.16	6.38	6.61	5.98
II.	12.46	12.01	11.46	9.64	9.22	9.89	6.93	7.01	7.33	8.52	8.21	7.96
III.	10.15	9.38	9.79	8.43	8.62	8.19	5.81	6.19	6.08	7.11	6.39	6.66
IV.	8.94	8.69	8.30	6.61	6.93	7.04	4.81	4.92	4.40	5.14	4.76	5.31
V.	9.96	10.36	10.18	9.22	9.60	8.96	7.10	7.03	7.48	8.32	7.94	8.01
VI.	10.10	11.02	10.76	9.11	10.32	8.28	7.09	7.51	7.92	7.08	8.39	8.69
VII.	9.75	10.40	10.31	9.29	8.51	9.56	7.21	7.03	7.42	7.49	7.88	8.50
VIII.	10.40	11.12	9.60	8.82	9.14	9.36	7.43	6.57	7.11	7.79	8.32	8.16
IX.	10.21	9.81	10.06	8.00	9.21	10.10	6.91	6.55	6.73	7.16	7.49	8.13
X.	11.90	11.34	11.68	9.42	9.31	8.99	7.01	7.19	6.77	7.82	8.17	8.42
XI.	9.95	9.62	9.49	8.36	8.01	6.98	6.31	6.42	6.06	6.22	6.56	5.03
XII.	10.81	10.42	11.24	8.71	8.49	8.22	7.23	7.07	7.46	7.10	6.82	6.30

TABLE IV-A.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media.	Soil No. 5			Soil No. 6			Soil No. 7			Soil No. 8		
I.	5.49	7.17	6.21	6.95	7.16	6.69	3.67	3.41	3.79	12.20	12.40	10.89
II.	6.44	5.56	5.85	8.67	9.12	8.82	4.61	4.52	4.63	12.06	11.83	12.81
III.	5.11	4.61	4.25	8.36	7.95	7.51	4.03	4.11	3.98	11.10	10.76	10.48
IV.	3.74	4.23	3.97	5.93	6.12	6.02	3.61	3.70	3.58	10.62	9.41	9.63
V.	5.85	5.92	5.42	6.21	6.78	6.91	3.92	3.46	3.21	11.06	11.82	11.32
VI.	7.25	6.65	5.56	7.67	7.48	6.98	4.58	4.61	4.32	11.26	11.91	12.16
X.	6.65	6.86	7.31	8.15	7.95	7.65	3.91	3.76	3.47	10.90	12.13	11.46
XI.	5.03	4.34	5.12	8.05	7.32	7.46	2.80	3.22	3.03	9.01	9.48	9.88
XII.	7.41	6.45	6.02	6.90	7.22	7.76	3.72	3.81	3.56	12.62	11.83	11.40
XIII.	7.16	7.03	6.75	8.22	9.40	8.83	4.41	4.58	4.13	12.14	11.66	12.49
XIV.	7.01	7.43	6.61	9.10	8.49	8.13	4.06	4.36	4.21	12.36	11.04	11.49
XV.	7.06	7.25	6.44	6.80	7.21	7.92	4.39	4.09	4.17	11.19	11.86	10.37
XVI.	6.88	6.36	6.52	7.22	7.39	7.83	4.52	4.16	3.94	12.45	11.64	11.21

TABLE V-A.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media.	Soil No. 9			Soil No. 10			Soil No. 11			Soil No. 12		
I.	6.61	6.06	6.44	4.12	4.58	4.33	4.91	4.95	4.87	7.80	6.39*	7.49
II.	7.48	7.29	7.71	4.41	4.11	4.08	6.13	6.30	6.27	9.68	9.77	9.81
VI.	6.87	7.06	7.39	4.01	4.36	4.44	5.70	6.04	5.83	9.21	9.08	9.59
XII.	6.38	6.86	6.52	3.99	4.13	4.22	4.87	5.13	5.34	8.33	8.40	8.11
XIII.	7.29	7.52	7.36	4.43	4.87	4.27	6.21	6.31	5.88	9.78	9.41	9.82

* Omitted from average.

TABLE VI.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media.	Soil No. 13			Soil No. 14			Soil No. 15			Soil No. 16		
II.	14.80	16.30	16.80	7.35	7.00	7.70	3.99	3.20	3.46	9.99	9.25	10.40
XIII.	9.80*	15.30	15.70	8.05	6.90	8.25	2.46	2.73	2.86	9.17	9.50	8.18
XVII.	14.60	13.80	Lost	7.75	9.60	7.96	3.00	3.12	2.86	7.04	7.50	7.86
XVIII.	16.40	18.20	Lost	7.90	8.20	10.30	3.86	3.99	*2.40	9.71	10.70	10.81
XIX.	14.80	16.60	14.30	7.10	7.70	Lost	2.60	2.50	Lost	9.37	9.64	7.83

* Omitted from average.

TABLE VII.

MILLIONS OF BACTERIA PER GRAM OF AIR-DRY SOIL.

Media.	Soil No. 17			Soil No. 18			Soil No. 19			Soil No. 20		
II.	14.70	12.70	13.30	6.41	6.82	6.22	2.30	1.94	2.40	5.40	5.35	4.33
XVIII.	15.65	17.10	15.30	6.96	7.01	7.34	2.61	2.18	2.46	5.60	4.47	4.61
XX.	15.60	14.30	14.95	7.10	6.78	7.25	3.46	2.93	3.07	4.67	4.54	5.74

THE INFLUENCE OF VARIOUS SALTS ON THE GROWTH OF SOYBEANS.*

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An experiment conducted by Dr. B. H. A. Groth, the object of which was to test the effect of various salts on the growth of soybeans *(Glycine hispida)* and prairie berries *(Solanum nigrum)*, was terminated in May, 1915. During the following summer this experiment was repeated in part, the same pots of soil being again employed without alteration. In preparation for the repeated experiment, the soil in each pot was thoroughly mixed and a sufficient quantity of tap water added to each culture to restore approximately its original water content (11.1+per cent on the air-dry basis). The series of pots from which a crop of soybeans had been harvested were again planted with soybeans. The series of cultures which had yielded a crop of prairie berries were again planted with prairie berry seeds. The latter, however, failed to germinate and this series of cultures was discontinued.

The soil used in the original experiment and also employed in the repeated experiment, consisted of a mixture of equal parts by weight of air-dry, white, sea-shore sand and rich garden soil. Each pot contained 4.5 kg. of this mixture.

In the original experiment, the salts added to the soil-sand mixture comprised the carbonates, chlorides, nitrates, phosphates, and sulphates of sodium, potassium, calcium, and ammonium, each used singly. The soluble salts were added to the soil in the form of solutions. The amount of salt in question required for a culture was dissolved in 500 c.c. of water and this solution was then added to 4.5 kg. of the soil and thoroughly mixed with it. The difficultly soluble salts were added to the soil in the powdered form. To 4.5 kg. of the soil was added the required amount of powdered salt in question and to the whole was then added 500 c.c. of water with thorough mixing.

Each salt was employed at five different concentrations, each of which represents a definite percentage value for all the salt radicals (theoretical atomic groups), Na_2O, K_2O, CaO, and NH_4 for the carbonates, and NO_3, PO_4, CO_3 and Cl for the nitrates, phosphates, sulphates, and chlorides, respectively. These five different concentrations of the salt radicals are 0.05, 0.10, 0.15, 0.20, and 0.30 per cent of the weight of the air-dry soil (4.5 kg. to each culture).

The cultures of this experiment may be divided into four groups with reference to the class of salts employed, the cultures containing

* Received for publication November 20, 1915.

the sodium, potassium, calcium, and ammonium salts,
prising the four groups. Each group of cultures employed five different salts (carbonates, chlorides, nitrates, phosphates, and sulphates) at five different concentrations, making a total of twenty-five cultures in each group. In addition each group contained a control culture consisting of the same soil-sand mixture as the other cultures, but containing no salts.

Table I gives the chemical formulae of the salts employed in each group of cultures, also the radicals upon which concentration calculations are based, and the actual weight in grams of the salt in each culture required to produce the various concentrations of the radicals in question.

TABLE I.

ACTUAL WEIGHT IN GRAMS OF THE SALTS EMPLOYED IN EACH CULTURE, CALCULATED FROM THE PER CENT VALUES OF THE SALT RADICAL CONCENTRATIONS.

Chemical formulae of salts	Salt radicals	Weight of salts required to produce salt radical concentrations of				
		0.05%	0.10%	0.15%	0.20%	0.30%
		gm.	gm.	gm.	gm.	gm.
Na_2CO_3	Na_2O	4.518	9.035	13.553	18.070	27.105
NaCl	Cl	3.710	7.420	11.130	14.840	22.260
$NaNO_3$	NO_3	3.083	6.165	9.248	12.330	18.495
Na_3PO_4	PO_4	9.005	18.009	27.014	36.018	54.027
Na_2SO_4	SO_3	3.950	7.900	11.850	15.800	23.700
K_2CO_3	K_2O	3.310	6.620	9.930	13.241	19.860
KCl	Cl	4.733	9.465	14.198	18.930	28.395
KNO_3	NO_3	3.670	7.340	11.010	14.680	22.020
K_3PO_4	PO_4	5.023	10.045	15.068	20.090	30.135
K_2SO_4	SO_3	4.845	9.690	14.535	19.380	29.070
$CaCO_3$	CaO	4.020	8.040	12.060	16.080	24.120
$CaCl_2$	Cl	3.525	7.050	10.575	14.100	21.150
$Ca(NO_3)_2$	NO_3	4.288	8.573	12.863	17.150	25.725
$Ca_3(PO_4)_2$	PO_4	3.666	7.332	10.998	14.664	21.996
$CaSO_4$	SO_3	4.838	9.675	14.513	19.350	29.025
$(NH_4)_2CO_3$	NH_4	6.185	12.370	18.555	24.740	37.110
NH_4Cl	Cl	3.393	6.785	10.178	13.570	20.355
NH_4NO_3	NO_3	2.905	5.810	8.715	11.620	17.430
$(NH_4)_2HPO_4$	PO_4	3.125	6.250	9.375	12.500	18.750
$(NH_4)_2SO_4$	SO_3	3.710	7.420	11.130	14.840	22.260

The seed used in this experiment was raised on the experiment plot of the botanical department of this station, and under favorable conditions yielded 96 per cent strong germination. The seeds were planted directly in the soil, ten seeds to each pot, at a depth of from 2 to 3 cm. Only five plants, however, were allowed to grow in each culture. In the higher concentrations the seeds in a number of cases failed to germinate and such cultures were discontinued.

The cultures were conducted in the experiment greenhouse during the time period from August 3 to September 20, 1915. The water lost

from each culture by transpiration and by evaporation from the soil surface was restored every second day by the method of weighing. The water added to the culture was, in each case, poured through a test tube open at both ends and placed vertically in the soil so as to extend about half way to the bottom of the pot. This prevented flooding of the surface of the soil. At the end of the time period of 48 days the plants were harvested. The tops were severed from the roots at the surface of the soil, placed in weighing bottles and dried for two days at a temperature of about 96° C. and from four to five hours longer at a temperature of from 102° C. to 104° C. The dry weights of the tops were then obtained in the usual way.

The numerical data of the yields of tops are presented in Table II. The dry weights of tops are given in this table relative to the average dry weights of tops of the control cultures taken as 1.00. The actual average dry weight, in grams, of these controls is given in parentheses in the table heading for the dry weight columns. The actual weight of any culture may be obtained by multiplying its relative weight by the actual weight of the average control culture. The blank spaces in the table indicate either failure of the seeds to germinate or failure of the seedlings to develop after germination had taken place.

TABLE II.

RELATIVE DRY WEIGHT OF SOYBEAN TOPS GROWN 48 DAYS IN SOIL SAND MIXTURE WITH FIVE DIFFERENT CONCENTRATIONS OF THE SALT RADICALS.

Chemical formulae of salts	Salt radicals	Dry weight of tops relative to the average dry weight of the controls (5.48 grams) taken as 1.00				
		0.05% concentration	0.10% concentration	0.15% concentration	0.20% concentration	0.30% concentration
Na_2CO_3	Na_2O	1.02	.93	....	....	....
NaCl	Cl	.49	.50	....	....	....
$NaNO_3$	NO_3	.84	.82	.98	....	....
Na_3PO_4	PO_4	.63	.64	....	....	....
Na_2SO_4	SO_3	.51	.45	.73	.52	....
K_2CO_3	K_2O	.98	.91	.47	....	....
KCl	Cl	.64	.55	....	....	....
KNO_3	NO_3	.80	.78	....	....	....
K_3PO_4	PO_4	.74	.52	....	....	....
K_2SO_4	SO_3	.54	.54	.62	.58	....
$CaCO_3$	CaO	1.28	1.33	1.35	1.20	.80
$CaCl_2$	Cl	.60	.57	....	....	....
$Ca(NO_3)_2$	NO_3	.73	.78	.69	.97	....
$Ca_3(PO_4)_2$	PO_4	.67	.62	.35	.30	.35
$CaSO_4$	SO_3	.56	.42	.68	.57	.32
$(NH_4)_2CO_3$	NH_4	.76	.72	.44	.65	....
NH_4Cl	Cl	.43	.34	....	....	....
NH_4NO_3	NO_3	.54	.43	....	....	....
$(NH_4)_2HPO_4$	PO_4	.51	.43	.33	.49	....
$(NH_4)_2SO_4$	SO_3	.62	.39	.41	....	....

From Table II it will be observed that only with 0.05 and 0.10 per cent concentrations did germination and development take place in all the cultures. With 0.15, 0.20, and 0.30 per cent concentrations the number of cultures which failed were 9, 12, and 17, respectively, out of a possible total of twenty cultures for each concentration. On the one hand, the failure in the germination or development of these cultures is undoubtedly related to the concentration of the salts in the soil solution, which in the high concentrations here employed, act osmotically to offer resistance to water entrance into the seeds and young roots, and, as might be expected, the number of failures increased as the concentration increased. On the other hand, the toxic action of the salts in each of these cultures may be a factor in the prevention or retardation of development. The former is an effect of the physical properties of the soil solution; the latter an effect of its chemical properties. It is, of course, entirely possible, and indeed probable, that these two factors acting at the same time are responsible for the results noted. To what extent failure or retardation in development of these cultures is due to one or the other of these two factors has not been determined.

Further inspection of Table II brings out the fact that only five cultures produced yields of tops superior to the average yield of the four control cultures. One each of these occurred with the 0.05, 0.10, 0.15, and 0.20 per cent concentrations of CaO, and the remaining one with the 0.05 per cent concentration of Na_2O. All other cultures produced yields inferior to the average yield of the control cultures. This retardation in the growth of the plants must be regarded as directly related to the unfavorable influence of the salts employed in these cultures, either by a toxic action affecting the life processes of the plants in a chemical way, or by giving rise to osmotic activities in the soil solutions resulting in too great resistance to water entrance into the roots in quantities adequate to supply the loss by transpiration and that used in the metabolic processes of the plant.

The relative toxic influences of the salts upon the growth of soybeans may be studied from the standpoint of relative dry weights as a criterion. With this point in view the dry weights of the tops, relative to the average control, of the plants grown in the cultures containing the sodium salts at the 0.05 per cent concentration were arranged in the order of their magnitudes, beginning with the highest. These form a rather uniformly decreasing series of numbers which were next plotted to form a graph shown as the heavy black line in Figure 1 (lower group of graphs). Here the abscissas were chosen arbitrarily to represent the different salts of the same base, the acid radicals of which are placed below. These acid radicals are the same for each group of salts. The

ordinates represent the relative dry weight values. With the same abscissas the corresponding dry weight values for the three remaining groups of cultures (potassium, calcium, and ammonium salts), at the 0.05 per cent concentration, were plotted, using the same scale for the ordinates, thus forming four graphs, each graph representing a single group of five cultures. The relative dry weight values for the four groups of cultures, all at the 0.10 per cent concentration, were plotted in a similar manner on the same sheet, using the same abscissas and the same ordinates. Curves of the dry weight values for the higher concentrations are not here presented since none of the groups is complete.

From Figure 1 it is at once clear that at the 0.05 and 0.10 per cent concentrations all the carbonates agree in showing higher dry weight yields than do any of the other salts in their respective groups. The dry weight values, arranged in the order of their magnitudes from the highest to the lowest yields, occur with the carbonates, nitrates, phosphates, sulphates, and chlorides respectively. At the 0.05 per cent concentration, however, ammonium sulphate produced a higher yield of tops than did the corresponding phosphate, and both calcium and potassium chlorides yielded higher dry weights of tops than did the corresponding sulphates. At the 0.10 per cent concentration, potassium sulphate yielded a slightly higher dry weight value than did the corresponding phosphate, and the chlorides of sodium, potassium, and calcium produced somewhat higher yields than did the corresponding sulphates, potassium chloride yielding also a slightly higher dry weight value than did the corresponding phosphate. The yields from cultures containing ammonium salts, in every instance are lower in value than the yields from the corresponding cultures of each of the other groups, excepting the yield from the culture containing ammonium sulphate at the 0.05 per cent concentration already noted.

With the 0.05 and 0.10 per cent concentrations, the cultures containing calcium carbonate show a markedly higher dry weight yield than does the average control. The culture containing sodium carbonate with the 0.05 per cent concentration also shows a slight improvement over the average control. The remaining cultures containing carbonates show yields somewhat lower than the average yields from the controls, and this becomes marked in the case of the cultures containing ammonium carbonate. The dry weight yields of all the other cultures with the 0.05 and 0.10 per cent concentrations here considered, fall far below the average dry weight of the controls. The relative differences in the degree of the toxic influence of the various salts employed, manifesting itself in retarded growth, are indicated by the gradual downward slope of all the graphs in Figure 1. It will be observed that the ammonium salts, judging from the criterion of dry weight yields, exert a markedly

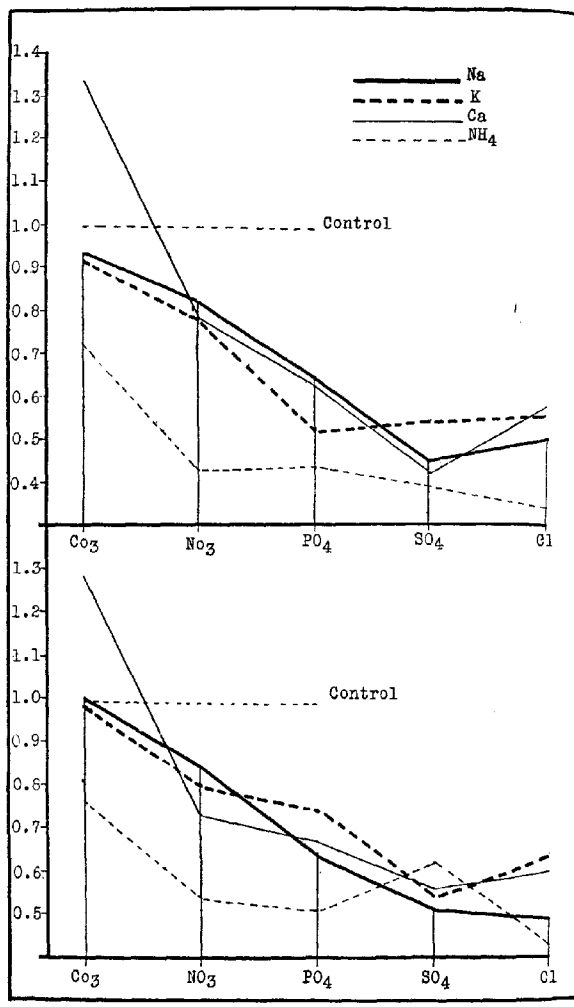

Figure 1—Graphs showing the relative dry weight values of soybeans grown in various cultures containing different salts.

greater toxic influence upon the growth of soybeans than do any of the corresponding salts of sodium, potassium, or calcium. There is, however, one notable exception in the case of ammonium sulphate with the 0.05 per cent concentration. Here the toxic influence, as indicated by the dry weight values, is somewhat less than that of the corresponding salts of the other basic elements (sodium, potassium, and calcium). With the 0.10 per cent concentration, however, this condition is reversed.

No marked differences excepting those of size occurred in the tops of the soybeans during the first twelve days of growth. At the end of this time, however, evidences of disturbed growth began to appear in certain cultures, first in the calcium phosphate cultures and later in all the phosphate cultures excepting those with the 0.05 per cent concentration. This disturbance manifested itself first in injury to the cotyledons. It consisted of a reddish-brown discoloration around the margins of these organs, gradually spreading toward the center. In severe cases the entire cotyledon became discolored and the death of the organ quickly ensued. In case of slight injury to the cotyledons, in addition to the marginal discoloration, reddish-brown spots also appeared at irregular intervals over the surface. In many cases the cotyledons completely recovered from this form of the injury, continuing normal during the remainder of the growth period. Usually an interval of several days elapsed between the time when the injury first manifested itself on the cotyledons and its appearance on the first pair of foliage leaves. The injury spread, in severe cases, to include all the leaves of the plant. In the leaves the disturbance appeared first as small, yellowish, translucent spots, which quickly took on the characteristic reddish-brown hue. These spots first appeared near the margin of the leaf and gradually increased in size and spread to cover the entire leaf, when death and falling of the leaf quickly followed. A foliage leaf once injured never recovered, however slight the injury may have been.

This injury occurred only with the cultures containing the phosphates and was most severe in the calcium phosphate cultures, all of which were dead at the time of harvesting. With the sodium, potassium, and ammonium cultures no injury occurred at the 0.05 per cent concentration, although at the 0.10 per cent concentration all the plants were severely injured, and with the higher concentrations all the sodium and potassium phosphate cultures failed, while with 0.15 and 0.20 per cent concentrations of ammonium phosphate the plants were dead at the time of harvesting.

The injury here described seems to be related directly to the phosphate salts. However, not sufficient data are at hand at the present time to warrant any definite conclusions. The reaction of soybean plants to-

ward the phosphate salts here dealt with singly and in combination with other salts, is at the present time the subject of further investigation.

Further evidences of disturbed growth appeared in the plants grown in the soil-sand mixture containing the ammonium salts. These plants as a whole were characterized by an unusually dark green coloration of the leaves, which may have been the result of an abundant supply of nitrogen, but here, at least, it is by no means an indication of a healthful condition. Not only had the plants grown in the lowest concentration (0.05 per cent) of the ammonium salts a decidedly deeper green coloration than had the plants from cultures containing the salts of sodium, potassium, or calcium, but the intensity of this coloration was correspondingly more pronounced as the concentration of the salts in the cultures was greater, and the dry weight yields correspondingly less. The plants from cultures containing ammonium salts were further characterized by exceedingly short leaf petioles, which gave the plants a stunted appearance.

The present experiments, as well as similar experiments immediately preceding this, have yielded little that might be construed as conclusive, with respect to the toxic or beneficial influences of the salts here employed, upon plant reactions. Nevertheless, these experiments have been a fruitful source of suggestions for constructive investigation.

FACTORS INFLUENCING THE PROTEIN CONTENT OF SOYBEANS,*

By Jacob G. Lipman, *Director*, and A. W. Blair, *Associate Soil Chemist, of the New Jersey Agricultural Experiment Stations.*†

This is a continuation of work that was begun in the summer of 1914, an account of which was given in the Annual Report of the Experiment Station for that year.‡ It was there pointed out that the protein content of soybeans may be considerably influenced by such factors as thickness of planting and date of harvesting, and also that the different varieties show considerable variation in protein content. Different fertilizing materials did not appear to make any material change in the protein content.

Series I.

Rate of Seeding.

The soil for this experiment was a Collington sand, slightly alkaline in reaction. Glazed earthenware pots, holding 20 pounds of soil each, were used. To the soil for each pot there was added 4 gm. of acid phosphate, and 2 gm. muriate of potash, in order that there should be no deficiency of minerals. On May 13th the pots were seeded to Swan soybeans, all being inoculated with an infusion made from soil in which soybeans had been grown. The beans were allowed to grow until the pods were well set, but before the leaves began to fall to any extent, they were harvested as forage.

The method of carrying out the experiment and the yield of dry matter and nitrogen are shown in Table I. With slight exception the increased rate of seeding gave increased returns in yield of dry matter, the thicker plantings giving about double the yield given with 2 to 8 plants per pot.

The percentage of nitrogen in the dry matter is slightly higher with the small number of plants to a pot than with the larger numbers. This is in accord with last year's results, though the differences for this year are somewhat more pronounced. In the matter of the total nitrogen recovered, the pots with 14 to 30 plants stand far ahead of those with 2 to 8 plants. This is due largely to the greater yield of dry matter of the former. With 20 to 30 plants a pot the yield of nitrogen is more than twice that with 2 to 4 plants a pot. As intimated in the previous report,

* Received for publication December 1, 1915.

† The field work in connection with the experiments here recorded was under the direction of Mr. L. K. Wilkins and the analyses were made by Mr. H. C. McLean, of the New Jersey Agricultural Experiment Station.

‡ New Jersey Agricultural Experiment Station, Annual Report, 1914, p. 240-245.

this would seem to indicate increased or intensified utilization of atmospheric nitrogen by means of symbiotic bacteria, with the thicker plantings. Certainly much more nitrogen is recovered from a given area, and if, as is generally conceded, much of this nitrogen is drawn from the atmosphere, there is good reason for thick seeding of soybeans.

TABLE I.

SOYBEANS—RATE OF SEEDING.

No.	Plants Per Pot	Dry Matter gm.		Per cent Nitrogen	Total Nitrogen mg.	
		Per Pot	Average		Per Pot	Average
1		24.5		3.440	843	
2	4 thinned to 2.....	27.8	26.15	3.321	924	884
3		19.5		3.264	636	
4	6 thinned to 4.....	26.2	22.85	3.272	858	747
5		39.8		3.272	1302	
6	12 thinned to 8.....	29.5	34.65	2.690	793	1048
7		41.0		2.986	1224	
8	18 thinned to 14....	50.8	45.90	3.253	1653	1439
9		58.8		2.995	1760	
10	24 thinned to 20....	56.5	57.65	3.035	1715	1738
11		53.7		2.848	1529	
12	30 thinned to 25....	62.5	58.10	3.006	1880	1705
13		63.2		2.986	1877	
14	36 thinned to 30....	63.4	63.30	2.867	1816	1847

SERIES II.

Can nodule formation be depressed?

It is generally believed that with an abundant supply of available nitrogen in the soil, leguminous crops do not draw as much of their nitrogen from the air as they do with an insufficient supply of soil nitrogen. With the hope of throwing further light on this question it was decided to try the effect of gradually increasing amounts of nitrogenous fertilizers in the growing of soybeans, to determine whether or not nodule formation may in this way be depressed. The experiment was carried out in glazed earthenware pots which held 20 pounds of white sand. To each pot the following were added: 4 gm. acid phosphate, 2 gm. potassium chloride, 10 gm. ground limestone, 0.5 gm. magnesium sulphate, and 0.25 gm. ferric sulphate. The pots were planted to Guelph soybeans on May 21st. The pots were inoculated by sprinkling with an infusion made with soil taken from a field where soybeans had grown successfully. The beans were allowed to grow until the pods were well filled. They then were harvested, roots and tops, the nodules counted, and the tops and roots weighed and prepared for analysis. Table II shows the plan for the special treatment and the weights of dry matter and nitrogen recov-

ered in both tops and roots. The amounts of ammonium sulphate and dried blood used are equivalent to 0.5 gm., 1 gm., 2 gm., and 4 gm. of nitrate of soda (15.93 per cent nitrogen), so that the results are comparable so far as the amount of nitrogen applied is concerned. Referring first to the part of the table dealing with the tops, it will be noted that, with slight exception, the applications of nitrogen have resulted in some increase in yield of dry matter, over the checks. With the dried blood there is a gradual increase in dry matter as the amount of blood applied is increased. The percentage of nitrogen in the dry matter is irregular and does not appear to be influenced greatly one way or the other.

TABLE II.

SOYBEAN VINES—CAN NODULE FORMATION BE DEPRESSED?

No.	Special Treatment	Dry Matter gm.		Per Cent Nitrogen	Total Nitrogen mg.		Increase over Check mg.
		Per Pot	Average		Per Pot	Average	
1		26.3		2.816	740		
2	No Nitrogen	20.5	23.40	3.241	664	702*	...
3		21.0		2.923	614		
4	.5 gm. Nitrate of Soda.	20.4	20.70	3.004	612	613	...
5		37.9		3.241	1229		
6	1 gm. Nitrate of Soda.	41.4	39.65	3.093	1281	1255	525
7		26.0		3.172	824		
8	2 gm. Nitrate of Soda.	39.0	32.50	3.153	1230	1027	297
9		30.6		3.104	950		
10	4 gm. Nitrate of Soda.	36.7	33.65	2.974	1091	1021	291
11		22.0		3.262	717		
12	No Nitrogen	24.0	23.00	3.162	759	738*	...
13		22.0		3.341	735		
14	.378 gm. Sul. Amm'a.	29.2	25.60	3.151	920	828	98
15		28.0		3.322	930		
16	.756 gm. Sul. Amm'a.	29.2	28.60	3.172	926	928	198
17		30.2		3.122	943		
18	1.512 gm. Sul. Amm'a.	37.0	33.60	3.151	1166	1055	325
19		26.5		2.955	782		
20	3.024 gm. Sul. Amm'a.	32.8	29.65	3.271	1074	928	198
21		18.2		3.182	579		
22	No Nitrogen	28.2	23.20	3.271	922	751*	...
23		25.5		3.202	817		
24	.6848 gm. Dried Bl'd.	24.0	24.75	3.202	768	793	63
25		26.5		3.103	822		
26	1.3697 gm. Dried Bl'd.	28.2	27.35	3.331	939	881	151
27		26.0		3.024	786		
28	2.7394 gm. Dried Bl'd.	30.2	28.10	3.222	972	879	149
29		29.0		2.658	771		
30	5.4788 gm. Dried Bl'd.	35.0	32.00	3.240	1134	952	222

What has been said with reference to the tops is generally true of the roots also. The percentage of nitrogen in the dry matter of the roots is irregular and not in proportion to the amount of nitrogen applied. The same is true with reference to the number of nodules.

* Averaged for check.

It therefore appears that, in sand culture at least, nodule formation is not depressed by applications of nitrogenous fertilizers. Evidently the plants did use some of the applied nitrogen, as evidenced by the gradual increase, in some cases, in the amount of nitrogen recovered, as the amount of nitrogen applied was increased. The excellent growth made by the checks, however, would lead to the belief that a large part of the nitrogen recovered, perhaps two-thirds or three-fourths, was secured from the atmosphere.

TABLE III.

SOYBEAN ROOTS—CAN NODULE FORMATION BE DEPRESSED?

No.	Special Treatment	No. of Nodules		Dry Matter gm.		Per Ct. Nit'gen	Total Nitrogen mg.		Inc. over Check mg.
		Per Pot	Ave'ge	Per Pot	Ave'ge		Per Pot	Ave'ge	
1		275		7.8		.600	46.8		(check)
2	No Nitrogen	290	282	3.3	5.55	.974	32.2	39.50*	39.90
3		243		6.8		.817	55.6		
4	.5 gm. Nitrate of Soda.	156	200	6.7	6.75	.699	46.8	51.20	11.30
5		331		7.6		.699	53.1		
6	1 gm. Nitrate of Soda.	321	326	9.1	8.35	.718	65.4	59.25	19.35
7		242		8.0		.551	44.1		
8	2 gm. Nitrate of Soda.	222	232	8.0	8.00	.688	55.0	49.55	9.65
9		142		6.8		.895	60.8		
10	4 gm. Nitrate of Soda.	199	170	8.0	7.40	.777	62.2	61.50	21.60
11		176		5.5		.826	45.4		(check)
12	No Nitrogen	309	242	7.1	6.30	.502	35.6	40.50*	39.90
13		158		5.8		.797	46.2		
14	.378 gm. Sul. Amm'a.	231	194	6.3	6.05	.669	42.2	44.20	4.30
15		253		6.1		.748	45.6		
16	.756 gm. Sul. Amm'a.	248	250	7.6	6.85	.590	44.8	45.20	5.30
17		238		7.7		.876	67.4		
18	1.512 gm. Sul. Amm'a.	432	335	8.2	7.95	.826	67.8	67.60	27.70
19		262		7.1		1.082	76.8		
20	3.024 gm. Sul. Amm'a.	250	256	10.5	8.80	.728	76.4	76.60	36.70
21		185		3.9		.974	38.0		(check)
22	No Nitrogen	253	219	9.1	6.50	.453	41.4	39.70*	39.90
23		223		5.8		.836	48.5		
24	.6848 gm. Dried Bl'd.	293	258	7.0	6.40	.748	52.4	50.45	10.55
25		205		7.7		.876	67.4		
26	1.3697 gm. Dried Bl'd.	318	261	6.5	7.10	.925	60.1	63.75	23.85
27		206		7.1		.708	50.3		
28	2.7394 gm. Dried Bl'd.	235	220	7.5	7.30	.826	62.0	56.15	16.25
29		179		3.6		.944	34.0		
30	5.4788 gm. Dried Bl'd.	381	280	8.1	5.85	.728	58.9	46.45	6.55

* Averaged for check.

SERIES III.

Varieties.

The soil used for this experiment was a loamy silt originally acid in reaction. The acidity was corrected by the use of a liberal application of ground limestone. No other fertilizing materials were used. The glazed earthenware pots used held 8 pounds of this soil. On May 29th the pots

were seeded, in duplicate, to fifteen different varieties of soybeans. All pots were inoculated with an infusion made from a soil taken from a plot on which soybeans had previously been grown. The varieties used and the yields of dry matter and nitrogen are indicated in Table IV.

TABLE IV.

VARIETIES OF SOYBEANS.

Number	Variety	Green vines and pods			Matured							
					Stems				Seeds			
		Dry Matter gm.	% Nitrogen	Total Nit'g'n mg.	Number	Dry Matter gm.	% Nitrogen	Nit. in stems mg.	Dry Matter gm.	% Nitrogen	Nit. in seeds mg.	Total Nit'g'n mg.
1	Guelph	24.2	3.046	737	2	10.5	.885	92.9	10.0	6.160	616.0	708.9
3	Ohio 9035	24.5	2.680	656	4	9.0	1.063	95.7	4.1	6.188	253.7	349.4
5	Swan	26.2	2.839	744	6	10.2	.935	95.4	6.7	6.533	437.7	533.1
7	Ebony	26.2	3.460	906	8	9.3	1.515	140.9	7.0	6.612	462.8	603.7
9	Tarheel	42.0	2.803	1177	10	16.0	.974	155.8	14.9	6.198	923.5	1079.3
11	Edna	24.6	2.978	732	12	6.9	1.181	81.5	7.0	5.925	414.8	496.3
13	Ito San	22.5	3.026	681	14	7.0	1.210	84.7	6.8	6.385	434.2	518.9
15	Black eyebrow	22.4	2.769	620	16	7.5	.767	57.5	9.0	6.660	599.4	656.9
17	Hollybrook	26.4	2.996	791	18	8.0	.826	66.1	9.0	6.775	609.8	675.9
19	Wilson	21.7	2.670	579	20	8.0	1.279	102.3	3.6	5.372	193.4	295.7
21	Manhattan	24.6	3.153	776	22	8.0	.679	54.3	9.8	6.809	667.3	721.6
23	Claud	23.0	2.871	660	24	9.3	1.092	101.6	8.1	5.205	421.6	523.2
25	Medium Yellow	29.8	2.512	749	26	9.2	1.004	92.4	7.0	5.952	416.6	509.0
27	Manchu	20.0	3.056	611	28	6.5	1.013	65.8	8.5	6.425	546.1	611.9
29	Baird	27.2	2.678	728	30	9.0	1.053	94.8	8.8	6.169	542.9	637.7

It should be pointed out that half the pots—the odd numbers—were harvested as forage, while the even numbers were left to ripen seed, and were harvested as seed and stalks separately. The leaves had largely disappeared from the stalks by the time the pods had ripened.

Of those harvested as forage nine varieties, Tarheel, Medium Yellow, Baird, Hollybrook, Ebony, Swan, Manhattan, Edna, and Guelph, in the order named, yielded more than 24 gm. of dry matter. All of these varieties also yielded more than 700 mg. of nitrogen. Tarheel gave the highest yields of dry matter and nitrogen, while Ebony stands second in the yield of nitrogen, and Medium Yellow second in the yield of dry matter. Six varieties, Ebony, Manhattan, Manchu, Guelph, Ito San, and Hollybrook, show 3 per cent or over of nitrogen in the dry matter. Four of these, Ebony, Guelph, Manhattan, and Hollybrook, stood considerably above 3 per cent in the 1914 test. Manchu and Ito San were not included in the experiment of last year. Of those harvested after seeds had ripened, Tarheel, Guelph and Swan lead in the yield of dry stalks, while Tarheel, Guelph and Manhattan lead in the production of seed. As might be expected, the percentage of nitrogen in the dry stalks is decidedly less than in the vines as harvested for forage. This is no doubt largely accounted for by the separation of the beans and by the loss of the leaves.

Five varieties, Manhattan, Hollybrook, Black Eyebrow, Ebony and Swan, show more than 6.5 per cent of nitrogen in the dry beans, and six varieties, Manchu, Ito San, Tarheel, Ohio 9035, Baird and Guelph, show between 6 and 6.5 per cent of nitrogen. Nearly all of these are likewise among those that yielded the highest percentage of nitrogen and the highest total nitrogen in the forage.

A word of caution should be given in regard to the Tarheel, which has shown up so well in this experiment. It is a late, slow-growing bean, and unless planted early and in good soil it would probably not mature seed. It makes a large growth of vines and if planted early would be good as a forage crop or as a green manure. Othewise it could not be recommended for central or northern New Jersey.

From the above it will be noted that the varieties here tested differ considerably both as to yield of dry forage and beans, and also as to percentage of nitrogen in the dry matter. For example, the lowest percentage of nitrogen in the dry beans is 5.21 and the highest 6.81, a difference of 1.6 per cent, which is equivalent to 10 per cent of protein.

The work should be repeated in duplicate, however, before definite conclusions on this point are arrived at.

Certain varieteis, as Guelph, Ohio 9035, Swan, Ebony, Tarheel (with the exceptions already noted), Hollybrook and Manhattan, seem to stand out prominently as giving a good yield of dry matter and a high percentage of nitrogen, and whether one is selecting the beans for a feeding material or for a green manure crop, this is important. In the case of a green manure crop, one of the high yielding varieties with a high nitrogen content will add to the soil far more nitrogen per acre than one that is low in both. In a field test in 1914* five varieties grown on limed plots gave an average of 6.7 per cent of nitrogen in the shelled beans, while the same varieties grown on unlimed plots gave an average of 6.2 per cent of nitrogen in the shelled beans.

Variety Test of Soybeans—Field Experiment.

Continuing the work of 1913 and 1914, an account of which was given in Bulletin No. 282 of this Station, fifteen varieties of soybeans were grown again this year on field plots varying in size from 1/80 to 1/20 of an acre.

All plots had previously been inoculated, and as in previous years acid phosphate was applied at the rate of 400 pounds and muriate of potash 100 (formerly 200) pounds per acre. In the spring of 1913 certain of the plots received a treatment of ground limestone at the rate of 2 tons per acre, while certain other plots did not receive the lime treatment. The soil is a loam to sandy loam, on which leguminous crops have been grown for some years.

* Factors Influencing the Protein Content of Soybeans. New Jersey Agricultural Experiment Station, Bulletin No. 282, p. 13.

CROP OF 1915.

The ground was broken and prepared about the last of April, and about the middle of May the fertilizers were applied and the different varieties planted in rows 33 inches apart. The germination was fair and the beans were cultivated during the season in the usual way.

The beans were harvested by pulling up the vines after the pods had matured and standing in cone shaped bunches (roots up) to dry out. By this method of harvesting the leaves are largely left on the field. When they were sufficiently dry they were weighed, stalks and pods together, threshed, and the shelled beans weighed and ground for analysis. The weights, calculated to the acre basis, and the percentage of nitrogen in the shelled beans are shown in Table V.

TABLE V.

YIELD OF DRY MATTER, AND NITROGEN CONTENT OF SOYBEANS, 1915.

(Calculated to acre basis.)

Variety	Dry Weight Vines and Pods, lbs.		Dry Weight Shelled Beans, lbs.		Per Cent Nitrogen Shelled Beans	
	Limed	Unlimed	Limed	Unlimed	Limed	Unlimed
Baird	....	....	...	...	6.868	.....
Black Eyebrow	2240	1360	680	376	6.480	6.129
Claud	3680	....	1064	...	5.887	.....
Ebony	2400	....	944	...	6.743	.....
Edna	2480	....	1240	...	6.354	.....
Guelph	1600	....	536	...	6.497	.....
Hollybrook	2280	1920	756	672	6.868	6.148
Ito San Plot 67	2400	....	860	...	6.547	.....
Manchu	2220	1380	1044	600	6.497	5.682
Manhattan	2600	....	748	...	5.916	.....
Medium Yellow	2960	1680	1088	792	6.264	5.720
Ohio 9035	2960	2080	1136	592	5.897	5.712
Swan	3480	1320	1278	564	6.663	5.964
Tarheel	4800	2240	616	272	6.178	6.062
Wilson	1800	....	984	...	5.923	.....
Average	2991*	1711	943*	553	6.407*	5.917

* The average for those varieties only that have corresponding unlimed plots.

For the limed sections Tarheel gave a yield of 4800 pounds of total dry matter, followed in order by Claud with a yield of 3680 pounds, and Swan with 3480 pounds. All other varieties fell below 3000 pounds of total dry matter. The highest yield of total dry matter on the unlimed sections was 2240 pounds for Tarheel, and the average for these sections was 1711 pounds as against an average of 2991 pounds on the corresponding limed sections. The largest yield of shelled beans on the limed sections was 1279 pounds from Swan, followed in order by Edna with a yield of 1240 pounds, Ohio 9035 with 1130 pounds, Medium Yellow with 1088 pounds, Claud with 1064 pounds, and Manchu with 1044 pounds. All other varieties gave a yield less than 1000 pounds. The highest yield

of shelled beans on the unlimed section was 792 pounds from Medium Yellow, and the second highest 672 pounds from Hollybrook. The average yield from these sections was 553 pounds as against an average of 943 pounds from the corresponding limed sections.

Here is a difference of 390 pounds of shelled beans (6.5 bushels) in favor of the limed sections. In addition to this the same plots yielded 1280 pounds more of dry stalks than the corresponding unlimed plots. These could be used in making manure or could be returned directly to the land to be plowed under.

The following varieties from the limed sections show a nitrogen content in the dry beans of 6.66 per cent or more: Swan, Hollybrook, Ebony, and Baird. The following show a nitrogen content of above 6 per cent, but less than 6.66 per cent: Ito San, Guelph, Manchu, Black Eyebrow, Edna, Medium Yellow and Tarheel. The highest percentage of nitrogen in those from the unlimed sections is found in the Hollybrook, 6.148 per cent. The average nitrogen content of the shelled beans from the unlimed section is 5.917 per cent and the average from the corresponding limed sections is 6.407, a difference of one-half per cent nitrogen, equivalent to 3 per cent protein, in favor of the limed sections.

The percentage of nitrogen in the dry beans this year is somewhat lower than in 1914, the average for that year being 6.205 per cent for the unlimed sections and 6.702 per cent for the corresponding limed sections. The average yield of shelled beans is higher this year than last.

Determinations of the nitrogen in the vines were not made, but it was shown in work reported one year ago that liming does increase the nitrogen content of vines. In the case of non-leguminous crops such nitrogen must come largely from commercial fertilizers and manures or from the store of soil organic matter, but in the case of a leguminous crop much of this nitrogen is taken from the air and thus becomes a definite contribution to the nitrogen store of the soil.

Of the 1915 crop, Black Eyebrow, Ito San, Manchu, Manhattan and Ebony matured in about 120 days from date of planting. Claud, Guelph, Ohio 9035, Wilson, Swan, Medium Yellow and Edna matured in about 135 days, while Tarheel and Hollybrook required 150 days, the former not having fully matured in this time.

In some cases those varieties that gave the largest yield of total dry matter also gave the largest yield of shelled beans. In other cases a low yield of total dry matter was accompanied by a rather high yield of shelled beans, as for example, Manchu. The reverse is true of the Tarheel, which is a slow growing late variety and should not be depended upon to mature seed. If, however, the growing season is long and the ultimate aim is to obtain a large amount of organic matter for soil improvement, this variety promises well. Swan and Claud are likewise rank growers.

DIASTASE ACTIVITY AND INVERTASE ACTIVITY OF BACTERIA.*

By George P. Koch,

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In the production of nitrogen compounds for consumption by plants, and in the decomposition of carbohydrates by bacteria in the soil, very complicated chemical reactions take place. Enzymes are secreted to effect certain changes; likewise acids, bases, and other compounds are formed. It is with these first mentioned properties that this paper primarily deals. It has been shown by a number of investigators that microörganisms during their life cycle secrete different enzymes. This study is, however, limited to the diastases and invertase.†

That bacteria have the property of secreting certain starch dissolving ferments was shown by Wortman (30) in 1882. Observing that bacteria were able to cause a change in starch paste and starch grains as well as in soluble starch, he concluded that the action of bacteria upon starch is through the same process, namely by a ferment such as diastase which is soluble in alcohol and water. In 1890 Fermi (6) recorded the examination of many species of bacteria for diastase and found that the Prioi's bacillius, Koch's *cholera vibrio, bacillus ramosus, bacillus megaterium* and a spirillum of cheese, gave a positive test for diastase. Cavazzani (3) in 1893 while working with an organism which he identified as probably being *bacillus maydis,* found that this organism had the ability to convert starch into glucose. That fungi as well as bacteria secrete a starch destroying enzyme was shown by Kohnstamm (17) in 1901. He studied the enzyme activity of wood destroying fungi and obtained a starch liquifying amylose from three of these.

That microörganisms secreted a sucrose-inverting ferment was already demonstrated by Borquelot (2). Later Beijerinck (1) in 1885 showed the inverting ability of *sacch. Kephir* and *sacch. Trycola.* He also showed that these organisms cause a change in cane as well as in grape sugar. It is recorded that Fermi (6) examined sixty-two microörganisms for inverting power, only two of which he found possessed this property. In 1890 Kellner, Mori and Nagaoka (14) wrote of the inverting ability of *eurotinus oryzae.* Sclavo (24) who in 1890 studied the biological properties of several bacteria, found that but few usually had the inverting power when grown upon a sugar-free nutrient boullion. Prob-

* Received for publication November 15, 1915.

† The thanks of the writer are expressed to Dr. J. G. Lipman for many suggestions which he has supplied throughout the study of this series of problems.

ably the most extensive work on the inversion of cane sugar by microörganisms was accomplished by Fermi and Montesano (9). These investigators attempted to find out what organisms cause the inversion of cane sugar. They also studied the influence of the reaction of the nutrient solution upon inversion by invertase, and the effect of the presence of the different sugars on inversion. Other factors concerning invertase that these investigators demonstrated were, the time at which invertase is present after the introduction of the sugar, what organisms secrete invertase in the absence of egg albumen, the retention of invertase on a porcelain filter, the influence of heat upon microbes, and the retention of invertase in dialyzing. Of the seventy microbes examined which had been grown on a sugar-free boullion the following organisms showed invertase activity: *bacillus megaterium, bacterium kiliense, proteus vulgaris, bacillus fluoresens liquifi,* a cholera vibrio, *vibrio Metschnikovii,* a white yeast, and a red yeast. It was found also that the quantative production of invertase was greater with the schizomycetes than with the schistomycetes.

More recent work is recorded by Grezes (10), who in 1912 showed that *Aspergillus niger* produced invertase in much larger quantities in the presence of sucrose and also that it still retained its inverting ability after it had been cultivated for sixty generations in a medium undesirable for invertase formation.

It seems very probable that some of the microörganisms of the soil secrete hydrolytic ferments during their life processes. Yet Fermi (8) in 1910 did not find any of the above mentioned enzymes in the soil samples which he examined. In 1911, however, Schreiner and Sullivan (23) demonstrated that hydrolytic, as well as proteolytic and cytolytic ferments exist in the soil. No doubt some of these ferments are the result of microbial activity and play an active rôle in carbohydrate destruction, and indirectly, in liberating other plant food elements.

Since it has been demonstrated by several investigators that bacteria do secrete diastases and have the property of inverting cane sugar, it was thought that more data bearing upon these points would not be amiss. Consequently these experiments were undertaken:

(1) To find out whether sufficient diastases and invertase were secreted by bacteria so that they could be quantitatively determined.

(2) To study the variation in the enzyme (diastase and invertase) secretion by organisms developed in culture solutions of different composition.

(3) To note the quantitative variation of enzyme secretion by bacteria at different periods.

(4) To ascertain if there is any direct correlation between enzyme secretion by bacteria and their property of decomposing proteins.

(5) To study the enzyme activities of various organisms and their ability to decompose proteins.

(6) To determine, if possible a correlation between the secretion of enzyme and the following: decomposition of proteins by bacteria, the property of the cultural solution to rotate the plane of polarized light, the percentages of reducing compounds, the formation of acid and the numbers of organisms; and also the inter-correlation which exists among the factors last named.

SECRETION OF ENZYMES, APPARENTLY DIASTASE AND INVERTASE, BY BACTERIA WHEN DEVELOPED IN DIFFERENT CULTURE SOLUTIONS.

The method of determining the diastase activity of extracts in which bacteria had been grown was the one proposed by Thatcher and Koch (28) for determining quantitatively the diastases of plant products. It was, however, not necessary to extract the enzymes since they were already in solution. Briefly, the method is as follows: The acidity of the enzmye solution is first determined by titrating a portion of the hot solutions with N/50 alkali. Then to 25 c.c. of a 10 per cent Lintner (18) soluble starch solution of known acidity, enough N/10 alkali is added so that upon introducing 20 c.c. of the extract the resulting solution, is of the optimium acidity for diastatic activity. This, as according to Effront (4), is 3 mg. of hydrochloric acid in 100 c.c. of solution. After the alkali has been added, 20 c.c. of the enzyme solution is introduced. The temperature of the solution is then quickly brought to 40° C. for a period of 30 minutes. Incubating at 40° C. for 30 minutes furnishes the standard conditions for diastatic activity recommended by Sherman, Kendall and Clark (25). At the expiration of the above mentioned time the enzyme activity was stopped by bringing the acidity of the solution to N/200 acid as recommended by Swanson and Calvin (27). After cooling the solution to 20°-22° C. the proteins were precipitated and removed from the solution. An aliquot of the filtered solution was taken for reduction with a definite volume of Fehling's solution and the amount of reduced copper determined by the iodine method as perfected by Peters (20).

With every determination a blank was carried through the same process in which the enzyme activity was stopped as soon as the 40° C. temperature was reached. The difference between the amount of copper reduced in the determination and that in the blank was considered due to the diastases secreted by the bacteria.

The determination of the quantity of invertase secreted by bacteria was similar to the method used for the diastases, with the exception that the enzymes were allowed to act upon a 10 per cent solution of sucrose at 55° C. Kjeldahl (16) concluded that at 52.5° C. the inversion process proceeds with greater rapidity and Effront (5) states, that the optimum

temperature according to different authors was found to be between 50° and 56° C. It was also found by Thatcher and Koch that 55° C. was the optimum temperature for invertase activity. The enzyme activity was stopped by boiling the solution 5 minutes. This method was found satisfactory by Thatcher and Koch* and in preliminary work which was performed in this laboratory.

In order to ascertain if diastases and invertase were secreted by bacteria in quantities sufficient to determine quantitatively and also to study the enzyme (apparently diastases and invertase) secreted by bacteria in culture solutions of different composition, culture solutions were inoculated with *bacterium mycoides* and *bacillus subtilis*. The boullion employed contained 10 gm. of peptone, 5 gm. of sodium chloride, and 5 gm. of Liebig's beef extract to a liter of distilled water. The acidity of the solution was corrected to 1 per cent or N/100 hydrochloric. A series of 99 c.c. portions of the above bouillon, a series of 99 c.c. of the same bouillon with an addition of 1 per cent sucrose, and a third containing 1 per cent Lintner's soluble starch were inoculated with 1 c.c. of a three-day-old culture of *bacterium mycoides*. In like manner portions of the different bouillons were inoculated with *bacillus subtillis*. After incubating for a period of 5 days at 25° C. the solutions were examined for diastase and invertase activity. The solutions were always tested for purity by plating 1 c.c. on "synthetic" agar (19). In every instance the heavy growth of bacteria was filtered off before making the enzyme determinations. The determinations were always made in duplicate.

TABLE I.

THE EXTRA-CELLULAR ENZYME ACTIVITY, APPARENTLY OF DIASTASE AND OF INVERTASE, SECRETED BY BACTERIA OF VARYING ACTIVITY IN CULTURE SOLUTIONS AFTER A FIVE-DAY INCUBATION PERIOD.

	Diastase gm. Cu. in 100 c.c. Solution		Invertase gm. Cu. in 100 c.c. Solution	
	Duplicate Determinations	Average	Duplicate Determinations	Average
Bacillus Subtilis.	.0000		.1152	
Bouillon	.0075	.0037	.1227	.1189
	.2531		.1753	
Sugar Bouilon	.2655	.2593	.1931	.1842
	.0812		.1504	
Starch Bouillon	.0710	.0761	.1253	.1378
Bacterium Mycoides.	.0176		.1604	
Bouillon	.0309	.0242	.1604	.1604
	.0000		.2381	
Sugar Bouilon	.0000	.0000	.2755	.2568
	.0251		.2705	
Starch Bouillon	.0201	.0226	.2705	.2705

* In unpublished data.

From the above table it is apparent that the bacteria have the property of secreting ferments in the various solutions in which they have developed, and the amount secreted is great enough for quantitative determination by the proposed methods. In the following discussions the enzyme activities will be referred to as diastase activity and invertase activity, although it has not been established beyond question that these alone are responsible for the changes effected in the solutions employed. That there is a considerable variation in the enzyme activity of the cultural solutions has been demonstrated. In the case of *bacterium mycoides* there was considerably more enzyme activity which was determined as invertase secreted in the starch bouillon than in the plain bouillon to which no soluble carbohydrates had been added. The sugar bouillon culture solution which was inoculated with *bacterium mycoides* seemed to show no diastase activity, but on the other hand, there was almost as much invert sugar formed in this solution as the result of activity, presumably of invertase, as there was in the case of starch bouillon. Quite the reverse was true with the cultures of *bacillus subtilis* as there was greater diastase activity and invertase activity in the sugar bouillon than in any of the other solutions inoculated with this organism. With regard to the relative amount of diatase and invertase secreted, with one exception there was considerably more invertase activity than diastase activity.

Protein Decomposition by Bacteria in its Possible Relation to Enzyme Activity at Different Periods.

Having found that the results of hydrolytic processes of bacteria could be determined by the method proposed, the writer considered it desirable to know at what time in the life cycle of the organism these hydrolytic ferments were secreted, and further if any possible relation existed between enzyme secretion and protein decomposition. Consequently a series of 99 c.c. portions of a 1 per cent sugar bouillon, sterilized and brought to 1 per cent acidity were inoculated with 1 c.c. of a three-day-old culture of *bacterium mycoides* and a similar series with *bacillus subtilis*. Fermi and Montesano (9), as well as other investigators found that a sugared bouillon was the most satisfactory medium for studying invertase activity. It was employed in the experiment cited above. On each of the nine consecutive days determinations for diastases and invertase activity and protein decomposition were made. The following method was employed for measuring protein decomposition. The 99 c.c. contents of the bacterial growth in the bouillon were placed in a copper flask, 100 c.c. distilled water added, and the ammonia distilled off by the addition of about 10 gm. of magnesium oxide. The distilling was regulated to deliver 100 c.c. in 40 minutes. This method was found to be satisfactory.

TABLE II.

THE VARIATION IN THE HYDROLYTIC PROCESSES AND THE PRC POSITION BY BACTERIA AT DIFFERENT PERIODS.

A.—BACTERIUM MYCOIDES.

Days after Inoculation	Diastase gm. Cu. in 100 c.c. Solution		Invertase gm. Cu. in 100 c.c. Solution		Ammonia produced in a 100 c.c. Solution, mg. N.	
	Duplicate Determinat'ns	Average	Duplicate Determinat'ns	Average	Duplicate Determinat'ns	Average
	.0101		.2909		1.17	
1	.0126	.0113	.2887	.2898	1.25	1.21
	.0405		.0354		1.20	
2	.0430	.0417	.0455	.0404	1.30	1.25
	.0304		—.0050		2.20	
3	.0254	.0279	.0000	—.0025	2.22	2.21
	—.0278		.3800		lost	
4	—.0227	—.0252	.3815	.3807	2.94	2.94
	.0050		—.1443		lost	
5	.0000	.0025	lost	—.1443	4.42	4.42
	.0000		.0254		8.97	
6	—.0025	—.0012	.0229	.0241	7.97	8.47
	—.0331		.0458		8.13	
7	lost	—.0331	.0636	.0547	7.25	7.69
	.0420		—.0356		10.50	
8	.0229	.0324	—.0611	—.0483	12.14	11.32
	.0458		—.0433		12.40	
9	.0458	.0458	—.0203	—.0318	10.36	11.38

B.—BACILLUS SUBTILIS.

Days after Inoculation	Diastase gm. Cu. in 100 c.c. Solution		Invertase gm. Cu. in 100 c.c. Solution		Ammonia produced in a 100 c.c. Solution, mg. N.	
	Duplicate Determinat'ns	Average	Duplicate Determinat'ns	Average	Duplicate Determinat'ns	Average
	.0607		.3087		.64	
1	.0607	.0607	.3016	.3051	.62	.63
	.0253		.0126		.81	
2	.0455	.0354	.0007	.0066	.85	.83
	—.0152		—.0152		1.44	
3	—.0101	—.0126	.0000	—.0076	1.52	1.48
	—.0177		.3853		2.26	
4	—.0227	—.0204	.3752	.3802	3.00	2.63
	.0050		—.1265		2.60	
5	—.0025	.0025	—.1342	—.1303	2.66	2.63
	.0433		.0101		4.61	
6	.0407	.0420	—.0076	.0012	3.65	4.13
	.0636		.0611		4.67	
7	.0458	.0547	.0791	.0701	5.09	4.88
	.0878		—.0254		6.88	
8	.0611	.0744	—.0203	—.0228	6.28	6.58
	.0839		—.0178		7.83	
9	.0687	.0763	—.0484	—.0331	8.41	8.12

Upon examining the above table it becomes apparent that the processes measured as diastase activity and invertase activity by bacteria in sugar bouillon vary greatly from day to day. Likewise there is a considerable difference in the secretion of these ferments by different organisms. Figures 1 and 2 express this to a much better advantage. In the case of *bacterium mycoides* a maximum diastase activity is reached on the second

day, after which there is a decrease. This fact is also recorded by Cavazzani (3), who states that on the following day the organism had lost much of its diastase activity. A negative determination of diastase appears on the fourth day, a fact which according to the data at hand, indicates that bacteria are not only capable of secreting hydrolytic ferments for the purpose of acting upon carbohydrates but likewise are able to produce a condition which prevents their hydrolysis. In order to simplify the terminology in this discussion, this negative activity, the exact nature of which has not been determined, will be arbitrarily referred to as resulting from a "contra" enzyme. The solutions showed slight diastase activity on the fifth day, a "contra" enzyme action again on the sixth and seventh, a marked activity on the eighth, and the maximum on the ninth day. In the case of the diastatic properties of *bacillus subtilis,* a marked activity was shown the first day, with a gradual decrease thereafter, so that on the third day a negative result was obtained. The fourth day shows a greater "contra" enzyme activity and then follows a gradual increase in extra-cellular diastase. On the ninth day, as in the case of *bacterium mycoides* the maximum activity was attained. Upon examining the text figure one sees that the diastase activity of the organisms varies considerably.

With regard to the extra-cellular invertase of these two bacteria, it is noted that in this respect the activities of these organisms were similar; as in both cases there was considerable invertase activity the first day with a decrease until a negligible degree was shown on the third day, the maximum on the fourth, and a "contra" enzyme action on the fifth day equal to one-third the activity on the previous day. Again there was considerable invertase activity on the seventh with a "contra" invertase activity on the eighth and ninth days.

With both organisms the maximum invertase activity was about six or seven times that of the diastases. Likewise the maximum "contra" invertase activity was four to six times that of the "contra" diastase activity. However, it is possible that these experiments were not repeated a sufficient number of times to demonstrate that these phenomena of "contra" enzyme activity always occur under conditions of the experiment.

Upon noting the text figures showing the decomposition of the proteins, i. e. formation of ammonia, it is apparent that with both organisms there was a gradual increase in the decomposition of the proteins up until the ninth day. From these data there seems to be no correlation between the secretion of the hydrolytic ferments and the production of ammonia due to the protein decomposition.

The Possible Relation of Enzyme Activities of Bacteria and their Ability to Decompose Proteins.

Since there was considerable irregularity in the secretion of hydrolytic ferments by *bacterium mycoides* and *bacillus subtilis* and since no

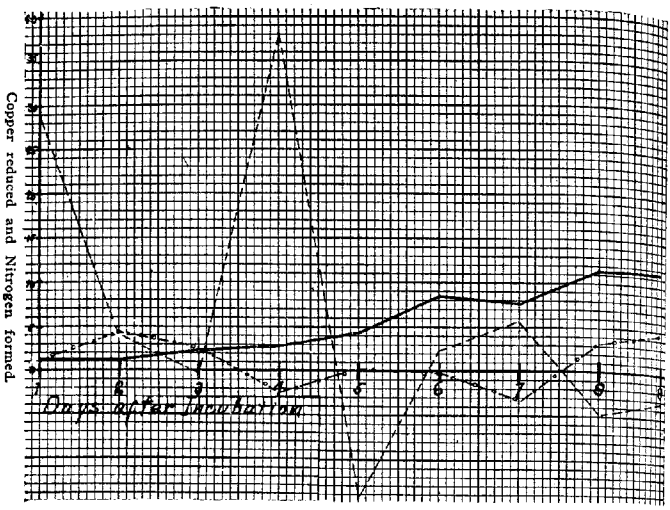


FIGURE 1.—THE VARIATION IN THE HYDROLYTIC PROCESSES AND THE PROTEIN DECOMPOSITION BY BACTERIUM MYCOIDES AT DIFFERENT PERIODS.

— — — — — Invertase—Each space represents .01 gm. Copper reduced.
— o — o — Diastase—Each space represents .01 gm. Copper reduced.
———— Ammonia—Each space represents 1 mg. Nitrogen.

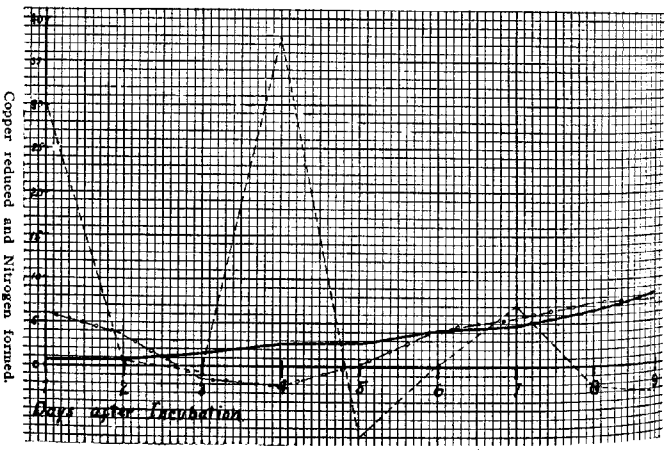


FIGURE 2.—THE VARIATION IN THE HYDROLYTIC PROCESSES AND THE PROTEIN DECOMPOSITION BY BACILLUS SUBTILIS AT DIFFERENT PERIODS.

— — — — — Invertase—Each space represents .01 gm. Copper reduced.*
— o — o — Diastase—Each space represents .01 gm. Copper reduced.
———— Ammonia—Each space represents 1 mg. Nitrogen.

* Errata:—The curve should read —.1303 on the fifth day, instead of —.0803.

correlation could be seen between the secretion of these ferments and protein decomposition, it was desirable to find out if any correlation existed between enzyme secretion and protein decomposition produced by other common soil organisms. In a manner similar to the methods previously used, sterile sugar bouillon was inoculated with three-day-old cultures of the different organisms and after being incubated for one day at 25° C. the inoculated culture solutions were examined for diastase and invertase activity and protein decomposition.

TABLE III.

THE ENZYME ACTIVITIES OF VARIOUS ORGANISMS, AND THE PROPERTY TO DECOMPOSE PROTEINS IN ONE DAY.

Kind of Organism	Diastase in 100 c.c. Solution, gm. Cu.		Invertase in 100 c.c. Solution, gm. Cu.		Ammonia produced in 100 c.c. mg. N.	
	Duplicate Determi'ns	Average	Duplicate Determi'ns	Average	Duplicate Determi'ns	Average
Bacterium Mycoides... 1.	.0000		.0335		5.80	
	.0038	.0019	.0360	.0347	5.94	5.87
" " .. 2.	.0388		.0258		.83	
	.0000	.0194	.0181	.0219	.85	.84
" " .. 3.	.0000		.0646		3.11	
	.0000	.0000	.0129	.0389	3.11	3.11
Bacillus Subtilis ..... 1.	.0155		—.0621		3.78	
	.0233	.0194	—.0697	—.0659	3.82	3.80
" " ..... 2.	.0190		—.0181		3.97	
	.0155	.0172	—.0362	—.0271	3.93	3.95
" " ..... 3.	—.0233		—.0258		.47	
	—.0310	—.0272	—.0206	—.0232	.45	.46
" " ..... 4.	.0794		—.2192		1.98	
	.0970	.0882	—.1965	—.2078	2.00	1.99
Bacillus Coli ..... 1.	—.0155		—.1419		3.26	
	—.0384	—.0219	—.1625	—.1522	3.26	3.26
" " ..... 2.	.0192		—.0335		3.59	
	.0116	.0154	—.0206	—.0270	3.69	3.64
Bacillus Cereus	.0078		.0181		2.66	
	—.0038	.0019	.0051	.0116	2.70	2.68
Bacillius Megaterium	—.0190		—.0776		.35	
	—.0697	—.0443	—.0335	—.0556	.37	.36
Bacterium Vulgaris	.0116		—.0466		4.57	
	.0310	.0213	—.0438	—.0452	4.57	4.57
Bacillus Cholera Suis	—.0384		lost		3.01	
	—.1163	—.0773	.0906	.0906	3.05	3.03
Bacillus Fluorescens Liqui...	.0038		.1865		.87	
	—.0116	—.0038	.1835	.1850	.91	.89
" " " ..	.0038		—.2096		2.62	
	—.0078	—.0019	—.2310	—.2203	2.62	2.62
Bacillus Vulgatus	.0384		lost		.37	
	.0697	.0540	.0517	.0517	.39	.38
Bacillus Proteus Vulgaris...	—.0116		—.1990		4.86	
	.0038	—.0038	—.2580	—.2285	4.96	4.91

The data in Table III show again that there is no direct correlation between secretion of hydrolytic "enzymes" and ammonia production. For instance, in the case of *bacterium mycoides* 2, which produced .84 mg. of ammonia, the diastatic activity is indicated by .0194 gm. of copper and the invertase activity by .0219 gm. of copper, while *bacillus fluorescens*

liquifaciens which produced almost the same quantity of ammonia, showed a diastase activity of —.0038, and invertase activity of +.1850 gm. of copper. Similar cases are noted upon the comparison of *bacillus cereus* with *bacillus fluorescens liqui* and *bacillus megaterium* with *bacillus vulgatus*. These organisms had a property of decomposing proteins similarly but their hydrolytic enzyme activities were entirely different. In like manner different pure cultures of the same species of organisms varied greatly in ammonia production as well as in enzyme secretion.

PROTEIN AND CARBOHYDRATE DECOMPOSITION BY BACTERIA IN ITS POSSIBLE RELATION TO ENZYME ACTIVITY.

Previous experimentation demonstrated the irregularity in the enzyme activity of the different bacteria and an absence of any possible correlation existing between the production of hydrolytic enzymes and protein decomposition. The next question which arises is whether there is any correlation between enzyme activity and the sugar content, presence of reducing compounds, formation of acid, numbers of organisms, and the amount of protein present. Two organisms, *bacillus coli* and *bacterium mycoides*, of entirely different morphological character and habitat, were studied for these properties. As in previous work sterile sugar bouillon solutions were inoculated with three-day-old cultures of the respective organisms. Daily determinations of the diastase and the invertase activities, optical properties, presence of "reducing" compounds, acid formation, numbers of organisms, and ammonia accumulations were made for a period of eight days.

The property of the solution of rotating the plane of polarized light was determined by first removing the proteins from the solution and then taking the reading directly by means of a saccharimeter.

The reducing compounds were determined by boiling an aliquot of the protein—free bacterial extract with a standard Fehling's solution and then applying the iodine titration method which was used in the enzyme procedure. The acid present was found by titrating an aliquot of the hot solution with N/50 alkali.

The bacterial counts were made upon synthetic agar on the fifth day after the plates were poured.

Upon examining Table IV and Figures 3 and 4, one sees at a glance that there is a considerable irregularity of hydrolytic enzyme secretion: on the second day there was considerable diastase activity which was almost similar to that tabulated in Table II. After a marked decrease in activity for two or three days the maximum was reached on the fifth day with *bacterium mycoides* and on the sixth with *bacillus coli*. With the exception of *bacillus coli*, which on the eighth day produced —.0301 gm. of copper, there were no other examples of "contra" diastase activity by these two organisms. As it was previously demonstrated,

TABLE IV.

DIASTASE AND INVERTASE SECRETION BY BACTERIA, THE DECOMPOSITION OF PROTEINS, THE ROTATION OF THE SOLUTION, THE FORMATION OF ACID AND THE NUMBERS OF ORGANISMS, PRESENT DAILY IN SUGAR BOUILLON INOCULATED WITH DIFFERENT ORGANISMS FOR A PERIOD OF EIGHT DAYS.*

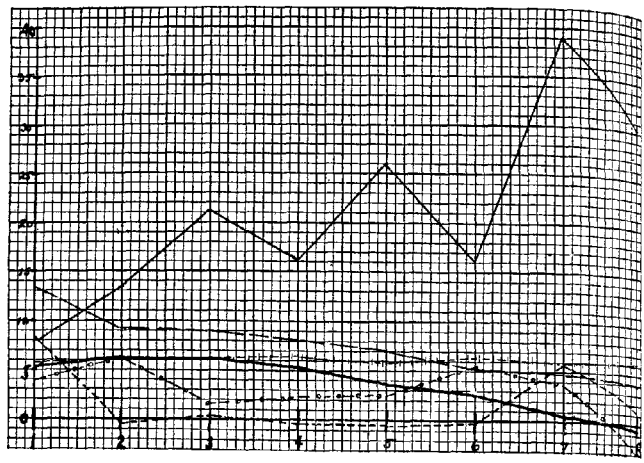
A.—BACILLUS COLI.

Days after Inoculation	Diastase in 100 c.c. Solution gm. Cu.		Invertase in 100 c.c. Solution gm. Cu.		Ammonia prod'd in 100 c.c. Sol., mg. N.		Saccharimeter readings. % of Sugar	Acid present in 100 c.c. gm. HCl	Numbers of organisms in 1 c.c.
	Duplic'te Determ's	Average	Duplic'te Determ's	Average	Duplic'te Determ's	Average			
1	.0292 .0488 .0624	.0390	.0390 .1341 —.0065	.0865	5.29 5.28 6.28	5.26	1.33	.0583	728,500,000
2	.0585 .0097	.0604	.0000 .0065	—.0032	6.40 6.14	6.34	.92	.0590	1,333,000,000
3	.0234 .0292	.0165	.0000 —.0234	.0033	6.14 5.45	6.14	.90	.0619	2,125,000,000
4	.0156 .0215	.0224	—.0130 —.0026	—.0182	5.53 3.69	5.49	.81	.0641	1,625,000,000
5	.0254 .0488	.0234	—.0039 —.0208	—.0033	3.79 2.40	3.74	.71	.0597	2,592,000,000
6	.0644 .0449	.0566	—.0247 .0637	—.0227	2.58 .61	2.49	.52	.0634	1,512,000,000
7	.0292 —.0527	.0370	.0521 .0168	.0579	.63 —.44	.62	.49	.0584	3,888,000,000
8	—.0075	—.0301	.0078	.0123	—.49	—.41	.39	.0616	3,024,000,000

B.—BACTERIUM MYCOIDES.

Days after Inoculation	Diastase in 100 c.c. Solution gm. Cu.		Invertase in 100 c.c. Solution gm. Cu.		Ammonia prod'd in 100 c.c. Sol., mg. N.		Saccharimeter readings. % of Sugar	Acid present in 100 c.c. gm. HCl	Numbers of organisms in 1 c.c.
	Duplic'te Determ's	Average	Duplic'te Determ's	Average	Duplic'te Determ's	Average			
1	.0175 .0156 .0565	.0165	.1054 .0872 .0182	.0963	7.36 7.36 13.71	7.36	1.33	.0561	82,150,000
2	.0546 .0390	.0556	.0130 —.0065	.0156	13.89 17.90	13.80	1.32	.0459	86,800,000
3	.0156 .0332	.0273	—.0156 .0091	—.0110	17.90 21.20	17.90	1.57	.0433	186,000,000
4	.0156 .0742	.0244	.0117 .0390	.0104	21.30 25.98	21.25	2.03	.0459	137,500,000
5	.0624 .0429	.0683	.0403 .0208	.0396	24.22 26.43	25.10	2.59	.0372	89,640,000
6	.0315 .0449	.0372	.0078 .0195	.0143	28.65 28.98	27.54	3.17	.0284	71,925,000
7	.0273 —.0156	.0376	.0065 .0585	.0130	30.82 31.36	29.90	3.26	.0251	54,000,000
8	.0195	.0019	.0390	.0487	32.00	31.68	3.30	.0277	54,000,000

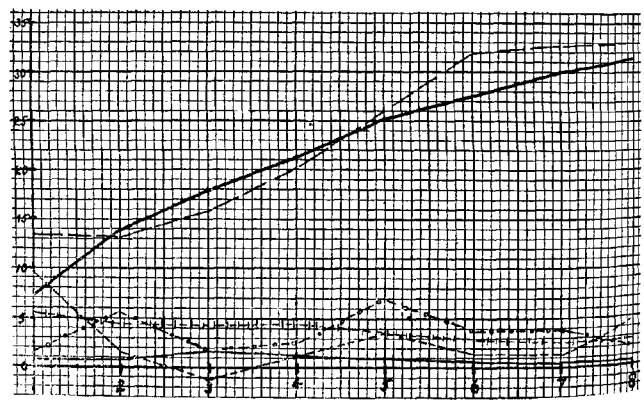
*In the case of the saccharimeter reading, an average of five determinations was recorded. cidity readings represent an average of four determinations; while the bacterial counts were ade in triplicate.



Days after Incubation.

FIGURE 3.—STUDIES ON THE ENZYME ACTIVITY OF BACILLUS COLI FOR A PERIOD OF EIGHT DAYS.

— — — — — Invertase—Each space represents .01 gm. Copper reduced.
— o — o — Diastase—Each space represents .01 gm. Copper reduced.
———— Ammonia—Each space represents 1 mg. Nitrogen.
—— —— Sacch. Readings—Each space represents .1% Sugar.
—|—|—|— Acid—Each space represents .01 gm. HCl.
———— Numbers of Organisms—Each space represents 100,000,000.



Days after Incubation.

FIGURE 4.—STUDIES ON THE ENZYME ACTIVITY OF BACTERIUM MYCOIDES FOR A PERIOD OF EIGHT DAYS.

— — — — — Invertase—Each space represents .01 gm. Copper reduced.
— o — o — Diastase—Each space represents .01 gm. Copper reduced.
———— Ammonia—Each space represents 1 mg. Nitrogen.
—— —— Sacch. Readings—Each space represents .1% Sugar.
—|—|—|— Acid—Each space represents .01 gm. HCl.
———— Numbers of Organisms—Each space represents 100,000,000.

there was a considerable amount of invertase secreted on the first day after inoculation. This was followed by a great decrease in activity, with a "contra" invertase activity followed by a steady increase and another decrease. On comparing the enzyme activity with the other products of the bacterial functions it is apparent that there is no correlation between them. With respect to the saccharimeter readings, the activities of the organisms were entirely different. In the solutions of *bacillus coli*, the saccharimeter readings were steadily on the decrease, while in the case of *bacterium mycoides* the reverse was true. The saccharimeter reading of solutions inoculated with *bacterium mycoides* on the first day was 1.33 per cent while on the eighth it was 3.30 per cent. The above shows that while the *bacillus coli* had utilized the greater percentage of sugar on the eighth day, *bacterium mycoides* had increased the sugar content or at least increased the saccharimeter reading at that time. A similar circumstance was observed in the protein decomposition, noted in the table above and in the text figure. In the case of *bacillus coli*, decomposition took place up to the second day while thereafter the protein formation was greater than the decomposition, so that on the eighth day there was slightly less free ammonia in the bacterial culture solution than there was in the original blank solution which had not been inoculated. This is no doubt due to the fact shown by Kendall (15) that the *bacillus coli* with the presence of a considerable amount of carbohydrates which are readily utilizable may protect the nitrogen compounds from attack by the organisms. In the case of *bacterium mycoides* there was a gradually increased protein decomposition up to and including the eighth day. It is very apparent that there is no direct correlation between the secretion of hydrolytic ferments, the saccharimeter readings and the protein decomposition. There seemed to be a direct correlation between utilization of carbohydrates, protein decomposition and the amount of acid formed. In the case of *bacillus coli* there was an almost steady increase of acid, showing that the carbohydrate decomposition was more rapid than the protein decomposition. In other words, more acid was liberated from carbohydrate decomposition than could be neutralized by the ammonia set free as the result of the protein decomposition. With *bacterium mycoides* the process was entirely reversed as in this case there was a continuous increase in the saccharimeter readings, the acid content steadily decreased while there was a continuous increase in ammonia. With both bacteria there was a gradual increase in numbers until the third day, after which there was a decrease in the *bacterium mycoides* cultures. This was probably due to the "dying out" of some of the organisms. The data in the text figure show a large secretion of invertase on the first day. In comparing this fact with the numbers of organisms present, it is probable that the rapid multiplication of the organisms caused the large secretion of invertase. In the case of *bacillus coli* this increase was from 1,199,700,000 to 72,850,000,000.

The determinations of the presence of reducing compounds demonstrated that the bacteria do not produce an excess of extra-cellular reducing substances. In all cases the blanks showed as much invert sugar as did the determinations. The fact that no invert sugar is present in a culture solution of sugared bouillon after the first or second days was pointed out by Fermi and Montesano (9). Hence the question at once arises, what part do these extra-cellular diastases play in the solution if they do not hydrolize organized compounds and make them more accessible for the organisms?

With regard to the acidity of the solution and the activity of disastases secreted by bacteria, it has already been found by Wortman (30), that the ferment secreted by the bacteria is able to act upon a solution containing starch when the solution is neutral and that in a weak acid solution the enzyme activity is increased. Likewise Fermi and Montesano (9) recorded that when the reaction of the sugar-free bouillon changes, some of the microbes lose this property and in slightly sugared bouillon all of them with the exception of *Vibrio Metschnikvii* retain their inverting property. It was found by Thompson and O'Sullivan (29) that invertase is very sensitive to acids. Hudson (11) observed that in alkaline solutions invertase shows no activity, while in the weakly acid solutions its enzymatic power reaches a maximum after which it decreases with increasing acidity. Since the acidity of these media was ten times greater than the degree of acidity which Effront (4) found to be optimum for diastatic activity, it seems that in solutions of as high concentration of hydrogen-ions as these (1 c.c. of the solution contained .0003645 gm. of hydrochloric acid) diastase formation would be impossible. Hudson (12) in 1908 found that the acidity for optimum invertase activity was 6N/10000 hydrochloric acid. Sorensen (26) in 1909 noted that the greatest activity was reached when the solution had the hydrogen-ion concentration of $10^{-4.4}$ to $10^{-4.6}$. Preliminary work by the writer demonstrated that there was little difference between the invertase activity when the acidity of the solution was 6N/10000 and that when the solution was 3N/10000 hydrochloric acid. Consequently there was almost twenty times as much acid in the solutions in which the bacteria developed as has been found to be the optimum acidity for invertase activity. Hence the question presents itself, do the extra-cellular enzymes secreted by the bacteria, whose optimum development is in solutions of N/100 acid (hydrochloric), function in the bacterial processes at such a high concentration of hydrogen-ions?

That protein compounds exhibit considerable influence upon the property of bacteria to secret hydrolytic ferments has been shown by Cavazzani (3) in his experiments with *bacillus maydis*. In the absence of protein he got only a trace of diastase activity, while in the presence of egg albumen, diastase activity equivalent to 0.115 gm. of glucose took place. In like manner Fermi and Montesano (9) showed that microbes

form invertase in the presence of egg albumen. Probably the most direct data available regarding the effect of the presence of protein upon enzyme activity is the work recorded by Rosenthaler (21). He shows that δ and σ emulsion exerts a protective action for the hydrolytic enzymes against alkali. Saito (22) also states, in work with *Aspergillus oryzae*, that nitrogenous bodies are contributing factors to the formation of diastase. Hence, no doubt, the presence of an abundance of protein compounds in these experiments exerted a protective influence against the attack of the acid. But in the case of *bacillus coli*, as shown in Table IV and in Figure 3, this organism increased the protein content of the medium after the third day, while the diastase activity and the invertase activity of the medium were not substantially different from those of *bacterium mycoides* which continually decomposed the proteins. Likewise the media in which *bacillus coli* developed continually increased in acid; but the harmful factors resulting from this increase in activity were probably neutralized by the increase in protein content. In the case of *bacterium mycoides* there was a gradual decrease in acid present and it is probable that the ammonia formed, as well as the presence of protein compounds, played the part of a protective agent against the acid.

The temperature at which the cultures were incubated, 25° C., was much lower than the optimum for activity of diastases and invertase. Several investigators have shown that the hydrolytic enzyme activity increases until the optimum is reached, beyond which point the activity is quickly destroyed. Thus at this temperature of incubation enzyme activity would take place, but not at its maximum rate. It therefore seems probable that the secreted hydrolytic ferments function in the bacterial processes even though, analytically, the conditions are not optimum for the activity of these enzymes.

SUMMARY.

The results obtained in the above experiments indicate that:

1. Enzymes determined as diastases and invertase are secreted by bacteria in culture solutions in amounts sufficient to be determined quantitatively.

2. There is considerable variation in the above mentioned activity of enzymes secreted by bacteria when they are developed in cultural solutions of different composition.

3. Enzyme activity (diastase and invertase) of bacteria as determined in this experiment is variable from day to day under conditions otherwise the same.

4. Bacteria appear to have the property of causing a factor which will prevent starch hydrolysis and sucrose inversion.

5. There seems to be no direct correlation between hydrolytic enzyme secretion and protein decomposition by bacteria.

6. Enzyme activity (diastase and invertase) of different species of bacteria varies greatly. Likewise there is a variation in enzyme activity of different cultures of the same species.

7. There is no direct correlation between hydrolytic enzyme secretion and the property of the solution to rotate the plane of polarized light, the percentage of reducing compounds present, the formation of acid and the number of organisms.

8. Bacteria have the property of increasing the rotatory power of a solution as they have of decreasing this property.

9. There is some evidence for a possible correlation between the utilization of the protein decomposition determined as ammonia and the formation of acid.

10. Bacteria do not produce in the solution a surplus of reducing compounds.

11. There is an increase in bacterial numbers up to the third day, after which with *bacillus coli* the numbers are irregular, while with *bacterium mycoides* there is a decrease.

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